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Neutrophil Responses to Mixed-Species Anaerobic Biofilms

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Neutrophil Responses to Mixed-Species Anaerobic Biofilms

by

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Abstract

Biofilms composed of anaerobic bacteria often result in persistent infections and chronic inflammation. Host immune cells have difficulties in clearing biofilm-related infections and this can result in unnecessary tissue damage due to the overproduction of proinflammatory mediators. The objective of this project was to generate mixed-species anaerobic biofilms composed of two opportunistic pathogens, *Fusobacterium necrophorum* and *Porphyromonas levii* and measure neutrophil responses. Neutrophils are a vital component of the innate immune system and the results show that neutrophils exposed to mixed-species planktonic bacteria exhibit a more extensive oxidative response than neutrophils exposed to biofilms composed of the same bacteria. The limited neutrophil response to biofilm bacteria may explain the reduced ability of the innate immune system to eradicate biofilm-associated infections. The results demonstrate that bacterial lipopolysaccharide plays a significant role in the stimulation of neutrophils, however the evidence points to the presence of other stimulatory molecules in the bacterial media.

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List of Abbreviations

ANOVA	Analysis of variance
AO	Acridine orange
ATCC	American Type Culture Collection
BHI	Brain heart infusion
CBD	Calgary biofilm device
CFU	Colony forming units
CPS	Capsule polysachharide
CSLM	Confocal scanning laser microscopy
DCF	Dichlorodihydrofluorescein diacetate
DMSO	Dimethyl sulfoxide
ECFs	Extracellular factors
EPS	Extracellular polysaccharide
EU/mL	Endotoxin units
FAA	Fastidious anaerobic agar
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HTA-Br	Hexadecyltrimethyl ammounium bromide
IκB	Inhibitor of nuclear factor
ICAM	Intracellular adhesion molecule 1
IKK	IκB kinase
IL-8	Interleukin-8
IRAK1	IL-1R-associated kinase 1
IRAK4	IL-1R-associated kinase 4
KW	Kruskal-Wallis non-parametric analaysis
LAL	Limulus amebocyte lysate
LBP	LPS binding protein
LDH	Lactate dehydrogenase
LKT	Leukotoxin
LPS	Lipopolysaccharide
MD-2	Myeloid differentiation factor-2
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary-response protein 88
NBT	Nitro-blue tetrazolium
NF-κB	Nuclear factor kappa-B
O-antigen	Oligosaccharide
pNA	Phenol nitroalanine
PAMPs	Pathogen associated molecular pattern
PMB	Polymyxin B
PRR	Pattern recognition receptor
R^2	Coefficient of determination
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RPM	Revolutions per minute
SEM	Standard error of the mean

Subsp.	Subspecies
TAK1	Transforming-growth-factor-β-activated kinase
TIR	Toll–IL-1–resistance domain
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TNF alpha	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6
VCAM	Vascular cell adhesion molecule

INTRODUCTION

1.1 Anaerobic bacteria

Anaerobic bacteria are ubiquitous in natural environments where oxygen is limited and represent the most prominent indigenous oral and gastrointestinal microflora in humans and animals (Goldstein and Finegold, 1984; Busch, 1984). They outnumber aerobic and facultative species by as much as 1000:1 in some locations and can behave as opportunistic pathogens if the conditions within the host are suitable (Brook, 2002; Jousimies-Somer *et al.*, 2002). Similar to aerobic bacteria, anaerobes can be divided into the broad categories of gram-positive or gram-negative based on the structure of their cellular membranes. Gram-positive bacteria stain purple due to the presence of a thick layer of peptidoglycan outside their outer membrane, whereas gram-negative bacteria do not retain the crystal violet Gram stain in their much thinner layer of peptidoglycan. This is a very general classification of bacteria and more advanced techniques for proper identification are constantly being developed (Schmitt *et al.,* 2013).

The anaerobic bacterial genera *Fusobacterium* and *Porphyromonas* consist of opportunistic bacteria that have been implicated in a wide variety of diseases affecting animals and humans (Darveau, 2010; Nagaraja *et al.,* 2005). Bacteria from these two genera live in close association with one another and are commonly isolated together from infections various infections that can cause calf diphtheria, hepatic abscesses and foot abscesses in ruminants (Tan *et al.,* 1996; Tadepalli *et al.,* 2008;). Species from *Fusobacterium* and *Porphyromonas* (eg. *F. nucleatum, P. gingivalis*) have also been shown to cause appendicitis and periodontitis in humans (Darveau, 2010; Tan *et al.,* 1996; Swidsinski *et al.,* 2011). *F. necrophorum* is one of the 13 known species of the

genus (Nagaraja *et al.,* 2005; Sigge *et al.,* 2007). There are two recognized subspecies in this genus: subsp. *necrophorum* and subsp. *funduliforme* (Kumar *et al.,* 2013). The focus of this project is on the subsp. *necrophorum,* as it is more virulent in animals due to the production of potent virulence factors and increased production of lipopolysaccharide (LPS) (Kumar *et al.*, 2013). *F. necrophorum* is a gram-negative, pleomorphic, rod-shaped anaerobe that can be isolated from the oral cavity and the gastrointestinal tract of humans and animals (Tadepalli *et al.,* 2008; Donelli *et al.*, 2012). As a primary or secondary etiological agent, *F. necrophorum* has many virulence factors that help evade host immune cells (Narayanan *et al.,* 2002). One example of this is the production and secretion of a potent leukotoxin (LKT) (Tan *et al.*, 1996; Narayanan *et al.,* 2002). At low concentrations this leukotoxin is known to cause induction of apoptosis and at higher concentrations results in necrotic cell death of bovine neutrophils (Narayanan *et al.,* 2002; Tan *et al.,* 1994). Strains that are unable to produce a leukotoxin exhibit a marked decrease in virulence (Emery *et al.,* 1985). Along with the leukotoxin *F. necrophorum* produces several other virulence factors such as a hemolysin that lyses erythrocytes (Miao *et al.,* 2010). Destroying host erythrocytes helps provide iron and create an anaerobic environment by preventing the transport of oxygen to a site of infection (Miao *et al.,* 2010). With this repertoire of virulence factors *F. necrophorum* can cause significant infections, especially in young or immunocompromised hosts (Ramirez *et al.,* 2003).

Due to its role in gingivitis, *Porphyromonas gingivalis* (*P. gingivalis*) is the most commonly studied species of the *Porphyromonas* genus. It produces a capsular polysaccharide (CPS) that enhances its pathogenic ability (Brunner *et al.*, 2010; Klein *et*

al., 2012). This virulence factor is capable reducing the production of pro-inflammatory mediators such as interleukin 8 (IL-8) and therefore plays an important role in immune cell avoidance (Darveau, 2010; Brunner *et al.,* 2010). The experiments in this project focus on the less-commonly known species *Porphyromonas levii* (*P. levii*). *P. levii* is a gram-negative, pigmented, coccobacillus-shaped obligate anaerobe that was originally isolated from the rumen of cattle (Summanen *et al.*, 2005). The black pigment that is observed in the colony morphology of *Porphyromonas* species is due to the acquisition of heme from the hemoglobin present in the growth media and the subsequent extracellular storage of heme on the outside of the bacterial cell wall (Byrne *et al.,* 2013). The retention of heme serves a crucial role in defense against destruction by hydrogen peroxide (due to the intrinsic catalase activity of heme) and also consumes oxygen, helping maintain an anaerobic microenvironment (Chen and Kuramitsu, 1999; Dashper *et al.,* 2004; Smalley *et al.,* 2000). *P. levii* also produces a potent immunoglobulin protease that can cleave host IgG antibodies and this potent virulence factor may help the bacteria avoid antibody-mediated phagocytosis by host neutrophils (Lobb *et al.,* 1999). The combination of these virulence factors (and others) allows *P. levii* to be an opportunistic pathogen, especially in anaerobic conditions like those observed in bovine interdigital phlegmon (foot rot) (Walter and Morck, 2002).

1.2 Biofilm formation

Up to 60% of the clinical infections treated with antibiotics involve the formation of biofilms (Fux et al., 2005). When bacteria grow as a biofilm they often show significant differences in gene expression when compared to their planktonic counterparts (Fux *et al.,* 2005). The genes that are up regulated in biofilm growth depend on the

species but they include genes for the production of EPS, cell signaling, membrane proteins and oxidative response proteins (Fux *et al.,* 2005; Sauer *et al.,* 2002). Biofilms form when free-floating (planktonic) bacteria are able to attach to a surface (Mah and O'Toole, 2001). The bacteria then produce an extracellular matrix consisting of polysaccharides, which enables the bacteria to firmly adhere to the surface (Mah and O'Toole, 2001). These extracellular polysaccharides hinder diffusion of antimicrobials and act as a physical barrier to host innate immune cells, allowing the bacteria to cause persistent infections (Campanac *et al.,* 2002; Lewis, 2001; Costerton *et al.,* 1999; Hoiby *et al.,* 2010). Diffusion of reactive oxidants such as the hydrogen peroxide produced by host immune cells can be so slow that they are inactivated before they get through the outer layer of the biofilm (Stewart, 1996; Bylund *et al.,* 2006). Antibiotics that are administered for the treatment of infections can also be inactivated before they penetrate the outer layers of a biofilm (Dibdin *et al.,* 1996). This diffusion barrier may create an environment that is advantageous for the development of random mutations conferring antibiotic resistance to the bacteria and certainly such constant low-level exposure to antibiotics may promote resistance development in the biofilm. Biofilm-associated bacteria exhibit environmental heterogeneity, meaning that different places within the biofilm have different concentrations of nutrients (Mah and O'Toole, 2001). At the center of a biofilm the oxygen and other nutrient concentrations can be 30 times less than the concentrations at the surface (Davies, 2003). This change in availability of nutrients causes cells near the center of the biofilm to exhibit a slow-growing state (Costerton *et al.,* 1999). It has been demonstrated that bacteria growing in this nutrient-restricted state are much less susceptible to antimicrobial agents because most antibiotics are targeted at

rapidly growing and dividing cells and do not affect dormant cells (Lewis, 2001). The difference in metabolic states of the cells within a biofilm ensures that at least some cells will survive antibiotic treatment (Costerton *et al.,* 1999). The frequency of mutation in biofilms has been shown to be much higher than in planktonic organisms, potentially leading to the development of antibiotic resistance more readily in biofilms than planktonics (Hoiby *et al.*, 2010). This phenomenon has been attributed to a hypermutable phenotype present in cells of a biofilm that causes mutations in the DNA repair pathways, leading to mutant phenotypes (Hoiby *et al.,* 2010). The pathways that are affected are the DNA mismatch repair pathway and the DNA oxidative lesions repair pathway (Hoiby *et al.*, 2010). A mutation in either of these pathways can potentially lead to increased antibiotic resistance (Hoiby *et al.,* 2010).

Recent research has shown that many species of bacteria can and will readily form biofilms, including the anaerobes (Donelli *et al.,* 2012). Previously, it was demonstrated that *F. necrophorum* can not only grow as a biofilm *in vitro* but can also form mixed-species biofilms with various other anaerobes and aerobes (eg. *Actinomyces spp., Staphylococcus spp.)* (Donelli *et al.,* 2007). When cultured together, species within the *Fusobacterium* and *Porphyromonas* genera grow in close association to one another on a variety of substrates such as rigid gas-permeable contact lenses and in the wells of tissue culture plates (Peyyala *et al.,* 2011; Periasamy and Kolenbrander, 2009). By living in close proximity to other bacteria, they present a much more formidable structure with a wide variety of virulence factors and this enables biofilms to persist in the natural environment. An example of this can be observed in cultures of *F. nucleatum* and *P. gingivalis,* where *F. nucleatum* generates carbon dioxide as a waste product which can

then be used by *P. gingivalis* (Diaz *et al.,* 2002). Carbon dioxide is essential for *P. gingivalis* survival and this cooperative metabolism allows *P. gingivalis* to exist in an environment that it wouldn't survive in on its own (Diaz *et al.,* 2002). The majority of biofilms found in the natural environment are polymicrobial (composed of multiple different species) and this provides numerous advantages to the bacteria (Wolcott *et al.,* 2013). Multiple species living together exhibit an increased rate of horizontal gene transfer and this allows the bacteria living within the biofilm to deal with environmental stresses more efficiently than a single species can (Madsen *et al.,* 2012). Bacteria that are cultured with other species often show an increase in pathogenicity, as observed with *F. nucleatum* and *P. gingivalis* (Wolcott *et al.,* 2013). These observations provide clear evidence for the reasons why bacteria aggregate and form polymicrobial communities and therefore mixed-species biofilms composed of *F. necrophorum* and *P.levii* are the focus of this project.

1.3 Neutrophil oxidative activity

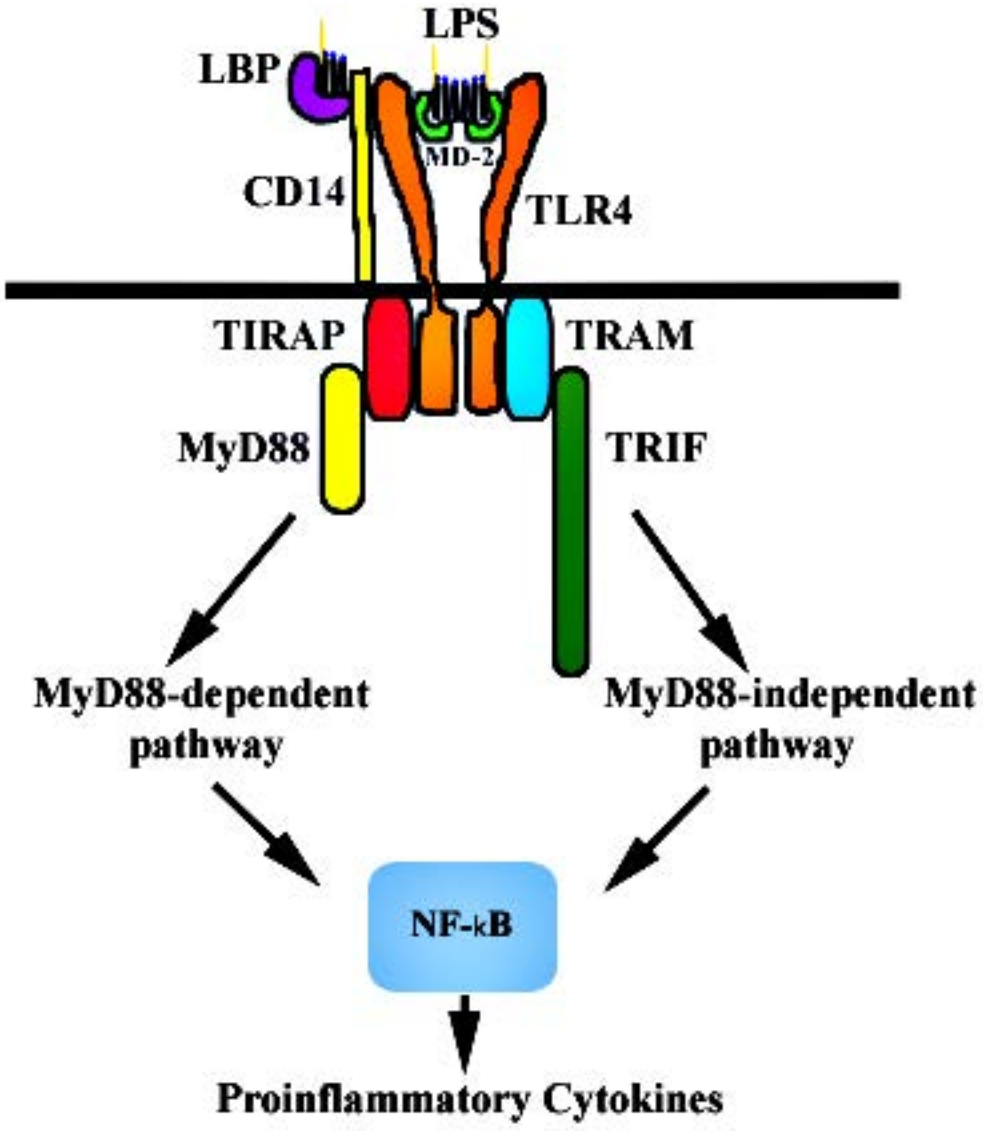
Neutrophils are a vital component of the innate immune system and provide the host organism with a first line of defence against invading pathogens (Voyich *et al.,* 2005). Neutrophils are phagocytic cells that are the first recruited cells to arrive at the scene of an infection to ingest and destroy microorganisms (DeLeo *et al.,* 1999). Neutrophil extravasation (migration from the peripheral circulation to a site of inflammation) involves a series of steps known as the leukocyte adhesion cascade. These steps include capture (tethering), rolling, adhesion and transmigration (Ley *et al.,* 2007; Herter and Zarbock 2012; Rigby and DeLeo 2012). Neutrophils are captured by E- and P-selectins expressed on the surface of endothelial cells, which bind to L-selectins present

on the surface of the neutrophils (Kuwano *et al.,* 2010). Transient interactions with the selectins allow the neutrophils to roll and search for chemotactic signals indicating tissue damage or invading pathogens (Ley *et al.,* 2007). Chemotactic molecules such as TNFα and IL-8 are released from resident tissue macrophages, mast cells, monocytes, epithelial and endothelial cells and bind to specific receptors on neutrophils, resulting in neutrophil activation (Rigby and DeLeo, 2012). Activation of neutrophils results in the up-regulation of integrins (CD11/CD18) on their cell surface, which bind to glycoprotein adhesion molecules (ICAM1 and VCAM1) present on endothelial cells and this results in the arrest of rolling (Rigby and DeLeo, 2012). Following firm adhesion, neutrophils can then transmigrate through the endothelium and follow a chemotactic gradient to the site of infection (Ley *et al.,* 2007).

Once neutrophils arrive at the site of infection, they can bind and ingest bacteria (phagocytosis). Neutrophils recognize various pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoprotein, flagellin or peptidoglycan; these can be either surface-bound to the bacteria or freely secreted (Durr *et al.,* 2006; Rigby and DeLeo, 2012; Danek, 2014). They detect PAMPs using various pattern recognition receptors (PRRs) expressed on their cell surface (Dower *et al.*, 2008). One example of a PRR present on the surface of a neutrophil is toll-like receptor 4 (TLR4), which binds to bacterial LPS and activates signal transduction pathways that ultimately lead to cytokine production to promote neutrophil phagocytosis, degranulation and the production of reactive oxygen species (ROS) (Figure 1; Lu *et al*., 2008; Akira and Takeda, 2004; DeLeo *et al.*, 1999). The activation of the transcription factor nuclear factor kappa-B (NF-κB) is a critical step in this pathway. The inactive form of NF-κB is sequestered in

Figure 1. Signal transduction at TLR4 in neutrophils activated by LPS

Neutrophils sense bacterial products such as LPS via their pattern recognition receptors. The LPS binding protein (LPB) binds to LPS and associates with CD14, which then promotes the transfer of the LPS to the TLR4/MD-2 receptor complex. Recognition of LPS results in a signal transduction cascade with the activation of NF-κB proinflammatory cytokines being produced as an end result. Modified from Lu *et al.*, 2008.


LPS
LBP
MD-2
CD14
TLR4
TIRAP
TRAM
MyD88
TRIF
MyD88-dependent pathway
MyD88-independent pathway
NF-kB
Proinflammatory Cytokines

the cytoplasm by the inhibitor of nuclear factor (IκB) and this needs to be phosphorylated to allow for the activation and subsequent movement of NF-κB into the nucleus (Akira and Takeda, 2004). Briefly, the binding of a ligand (such as LPS) to the TLR causes the association of myeloid differentiation factor 88 (MyD88) with the Toll–IL-1–resistance (TIR) domain on the cytoplasmic side of the membrane (Akira and Takeda, 2004; Fitzgerald *et al.,* 2003). MyD88 is essential for TLR signaling and promotes the association of two protein kinases, IL-1R–associated kinase 1 and 4 (IRAK1 and IRAK4) (Fitzgerald *et al.,* 2003). IRAK4 phosphorylates IRAK1, which creates a binding site for tumor necrosis factor receptor-associated factor 6 (TRAF6) (Kindt *et al.,* 2007). This allows for the activation of transforming-growth-factor-β-activated kinase (TAK1), which phosphorylates IκB kinase (IKK) (Kindt *et al.*, 2007). This is a crucial step because IKK phosphorylates the inhibitor of nuclear factor (IκB), causing it to release NF-κB (Kindt *et al.,* 2007). Following the release from IκB, NF-κB is free to translocate into the nucleus, where it binds to the NF-κB-binding motif and induces the expression of NF-κB-dependent genes (Kindt *et al.,* 2007; Akira and Takeda, 2004). The end result is the generation of proinflammatory cytokines, chemokines, and other effectors of the innate immune system (Kindt *et al.,* 2007).

There are many adaptor molecules involved in the signaling pathway, making it a complex and tightly regulated system (Lu *et al.,* 2008; Fitzgerald *et al.,* 2003). Improper regulation of this pathway can cause serious problems, including acute sepsis and chronic inflammation (Lu *et al.*, 2008). There are many other PRRs present on the surface of neutrophils that aid in the detection of foreign pathogens and these are not limited to the detection of bacterial products. For example TLR3 detects double-stranded viral DNA,

whereas TLR9 is dedicated to the detection of unmethylated CpG motifs present in bacterial and viral DNA (Alexopoulou *et al.,* 2001; Bauer *et al.,* 2001). Collectively, the PRRs located on immune cells allow the host to non-specifically detect a significant portion of the nucleic acids, lipids, carbohydrates and peptides expressed by various pathogens present in any environment (Trinchieri *et al.,* 2007).

This project focuses on the production of ROS by neutrophils as an indication of neutrophil activation. ROS are produced in neutrophils by the action of a membrane-bound NADPH-oxidase (Bylund *et al.,* 2006). The enzyme uses electrons from cytoplasmic NADPH to generate the superoxide ion from intracellular oxygen and this then dismutates to oxygen and hydrogen peroxide (Clark 1999; Karlsson *et al.,* 2002; Bylund *et al.,* 2006). The hydrogen peroxide is free to diffuse across biological membranes and interacts with myeloperoxidase (MPO) to generate the strongest forms of ROS (Bylund *et al.,* 2006). These ROS are very effective antimicrobial agents (Roos *et al.,* 2003). The production of ROS is just one of the mechanisms used by the neutrophils to destroy phagocytized bacterial cells, they can also produce reactive nitrogen species (RNS) and release numerous other cytokines and chemokines as part of their antimicrobial activity (Voyich *et al.,* 2005). They also produce peptides and proteases such as α-defensins, lysozyme and elastase that act as oxygen-independent antimicrobial molecules (Rigby and DeLeo, 2007).

1.4 Biofilms, neutrophils and persistent infections

More than half of the infectious diseases observed in the general population are due to commensal bacteria found in the environment and biofilm formation tends to play a major role in these types of infections (Costerton *et al.,* 1999). Biofilm-associated

infections tend to be persistent or chronic and this can be attributed to the numerous advantages that biofilm growth offers over planktonic bacteria such as the production of EPS and the increased expression of various proteins (Fux *et al.,* 2005). Biofilm-associated infections tend to slowly lead to tissue damage caused by the overproduction of proinflammatory molecules by frustrated neutrophils as observed in cystic fibrosis and periodontal disease (Cekici, 2014; Hassett *et al.,* 2010). Neutrophils can migrate to the surface of biofilms and phagocytize immature biofilms of *Staphyloccocus aureus* (Gunther *et al.,* 2009). However, the interaction of neutrophils with mature, well-established biofilms often results in less effective clearance, persistent infection and chronic inflammation (Hannan *et al.,* 2010; Gunther *et al.,* 2009). *In vivo* experiments have indicated that neutrophils can migrate within biofilms and actually become trapped inside, acting as a barrier to other host immune cells (Buret *et al.,* 1991). It is not well understood why neutrophils are incapable of clearing these biofilms but there is a consensus that mechanical difficulties of migrating through the extracellular matrix only play a partial role in the process (Hoiby *et al.*, 2010; Gunther *et al.,* 2009).

Bacterial infections are often treated with antibiotics and the selection of an appropriate antibiotic can impact the ability to effectively clear biofilms (Tre-Hardy *et al.,* 2010). Certain types of antibiotics, such as the macrolides have been shown to have immunomodulatory effects on the function of neutrophils (Buret, 2010; Fischer *et al.,* 2011; Chin *et al.,* 2000; Lee *et al.,* 2004). Macrolides are a class of antibiotic that contain a lactone ring and inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit (Tenson *et al.*, 2003). Some macrolide antibiotics (eg. Tilmicosin) can be internalized by neutrophils and have anti-inflammatory effects such as induction of

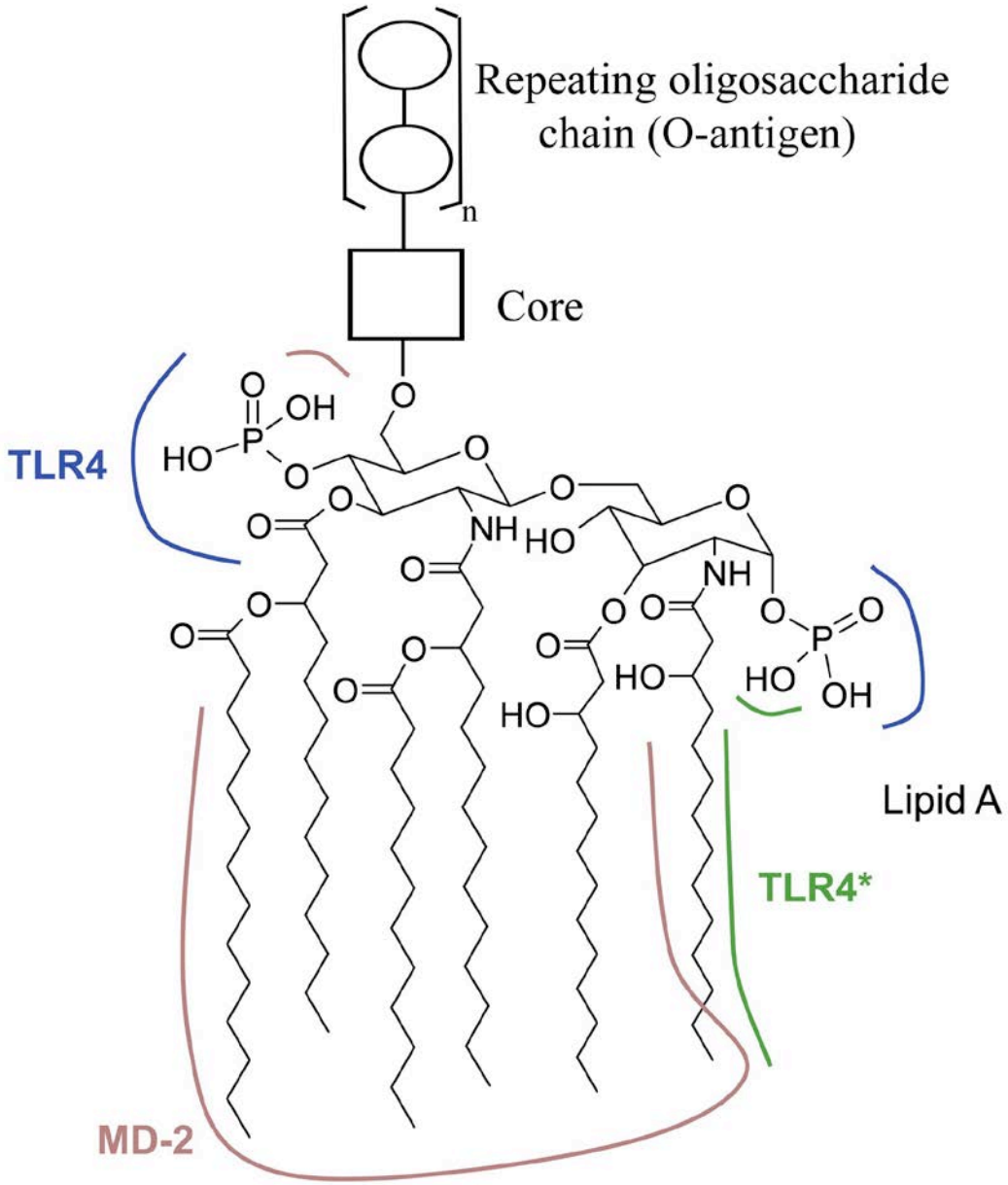
neutrophil apoptosis (Buret, 2010). This alteration of neutrophil function to reduce inflammation and avoid uncontrolled neutrophil death may be important when treating biofilm-mediated disease (Buret, 2010). Limiting the chronic inflammation may provide an advantage to using macrolides compared to other antibiotics such as the tetracyclines when treating persistent infections (Tre-Hardy *et al.,* 2010; Buret, 2010).

1.5 LPS and extracellular factors

Unlike gram-positive bacteria, which have a thick peptidoglycan layer outside their cellular membrane, gram-negatives contain a thin-peptidoglycan layer outside the cell membrane that is covered by an outer membrane (White, 2007). This outer membrane is mainly composed of LPS, phospholipids and proteins (White, 2007). LPS is a portion of the gram-negative outer membrane that consists of three regions: a hydrophobic lipid A that has a backbone composed of two glucosamine residues, the core, and a repeating oligosaccharide, which is often called the O-antigen (Figure 2; Trent *et al.,* 2006; Ray *et al.*, 2013). The O-antigen is made up of repeating units of sugars and the composition varies significantly among different strains of bacteria (White, 2007). The lipid A portion is embedded in the outer membrane with the core and oligosaccharide chain extending into the surrounding milieu (White, 2007). The LPS serves as a permeability barrier to hydrophobic compounds such as antibiotics and bile salts, allowing gram-negative bacteria to thrive in places like the intestine (White, 2007). LPS is detected by neutrophils and other host immune cells via the interaction of TLR4 with the lipid A component of LPS, causing neutrophil stimulation, chemotaxis and subsequent production of ROS (Poltorak *et al.,* 1999; Ray *et al*., 2013; Schletter *et al.,*

Figure 2. Structure of LPS

General molecular structure of LPS showing the lipid A region, core and O-antigen. The lipid A region is embedded into the outer membrane, and the highly variable O-antigen extends into the surrounding medium. The bioactive sites of lipid A that are recognized by TLR4 on neutrophils are also highlighted. Modified from Ray *et al.,* 2013.


Repeating oligosaccharide chain (O-antigen)
n
Core
TLR4
HO
OH
NH
HO
NH
HO
HO
HO
OH
Lipid A
TLR4*
MD-2

1995; DeLeo *et al.,* 1999). LPS can also affect the host immune response via the activation of the complement system (Jensen *et al.,* 1993).

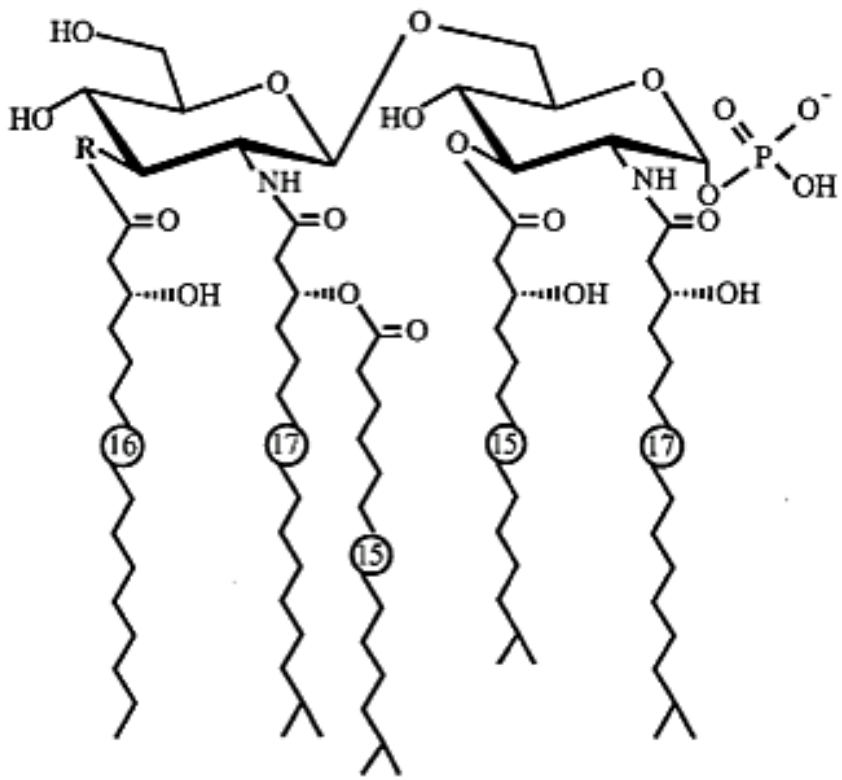
The lipid A portion of LPS is responsible for its toxicity (Caroff *et al.,* 2002). It was originally thought that the lipid A portion was highly conserved amongst different species of bacteria, however recent research indicates that it is more variable than previously thought (Figure 3; Park *et al.,* 2009; Trent *et al.,* 2006; Kato *et al.,* 1998; Kumada *et al.,* 1995). The structure of the lipid A component has been determined for many species and modifications to the lipid A component may help gram-negative bacteria avoid detection by TLR4 on host immune cells (Trent *et al.,* 2006).

Others have shown that there are some significant differences in the amount of LPS produced by bacteria living as a biofilm compared to their planktonic counterparts. In fact, LPS itself plays a major role in the formation of a biofilm (Chatterjee and Chaudhuri, 2006). Yeom and colleagues observed that biofilm-forming *E. coli* expressed more LPS synthesis genes and a higher level of expression of certain outer membrane pore genes (eg. *ompC*), resulting in a greater amount of LPS in biofilm-growth compared to planktonics (Yeom *et al.,* 2012). Gram-negative bacteria (*Pseudomonas aeruginosa)* growing as a biofilm have been shown to produce LPS that is structurally distinct from their planktonic counterparts. The LPS produced by biofilms of *P. aeruginosa* tends to have a lower molecular mass than the LPS produced by planktonic bacteria (Ciornei *et al.*, 2009). Furthermore, the lipid A portion of LPS is reversibly modified from the planktonic version when bacteria are grown as a biofilm and it has been demonstrated that biofilm-LPS induces the production of more proinflammatory cytokines than planktonic-LPS (Ciornei *et al.,* 2009). Such studies have not been performed on cultures of

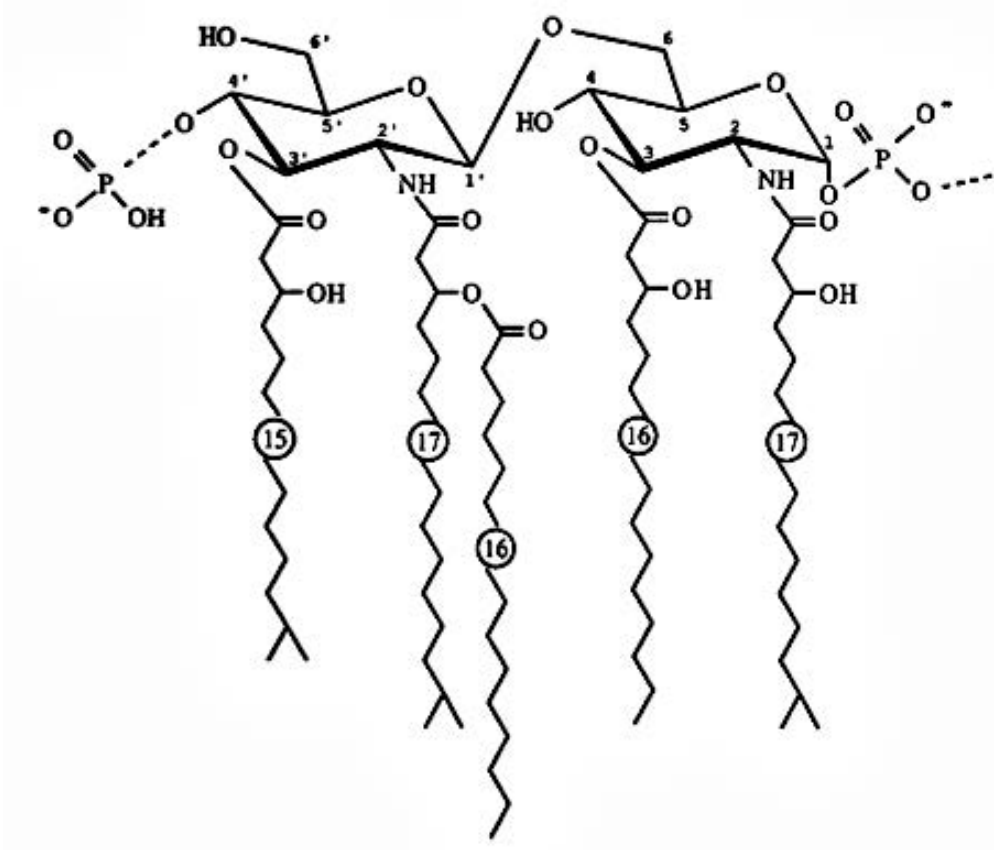
Figure 3. Examples of the various lipid A components of LPS from different gram-negative bacterial species

The structure of lipid A from two species of gram-negative bacteria. **A.** *Flavobacterium meningosepticum* **B.** *Porphyromonas gingivalis.* Examination of the molecular structure of LPS from various species shows a dramatic difference in the lipid A moiety depending on the species. Modified from Kato *et al.,* 1998; Kumada *et al.,* 1995.

A.



B.



F. necrophorum and *P. levii,* therefore the quantity of LPS generated by biofilms composed of these two bacteria was assessed in this study.

LPS can also promote the survival of neutrophils and delay the onset of programmed cell death (neutrophil apoptosis) (Yamamoto *et al.,* 1993; Lee *et al.,* 1993). Neutrophils constitutively undergo apoptosis and this is a critical step for the resolution of an inflammatory response, however some molecules (eg. granulocyte-macrophage colony-stimulating factor (GM-CSF) and LPS) are capable of altering the level of apoptosis in neutrophil populations (Collota *et al.,* 1992; Brach *et al.,* 1992). Neutrophils that were incubated with LPS prior to exposure to ultraviolet light showed a significant decrease in the amount of apoptosis compared to untreated neutrophils (Sweeney *et al.,* 1997). The mechanism of neutrophil survival is based on TLR4 activation and subsequent signaling through the NF-κB and the mitogen-activated protein kinase cascades (Figure 1; Sabroe *et al.,* 2003; Hachiya *et al.,* 1995).

This project focuses on the effect of extracellular LPS on neutrophils; however, there are numerous other molecules present in the growth media from mixed-species planktonics and biofilms that will need to be considered in the future (such as the *F. necrophorum* leukotoxin, peptidoglycan, various hemolysins and short-chain fatty acids (SCFA)) (Carretta *et al.,* 2012; Mills *et al.,* 2006; Nagaraja *et al.,* 2005). It is likely that a number of extracellular compounds will be able to interact with the various surface receptors expressed by neutrophils to detect PAMPs and a combination of these stimuli will result in neutrophil activation.

1.6 Hypotheses

We hypothesize that:

1. Mixed-species biofilms composed of *F. necrophorum* and *P. levii* can be generated and used to assess neutrophil responses.
2. Growth medium containing extracellular factors (ECFs) from biofilm bacteria will contain more LPS than growth medium containing ECFs from planktonic bacteria and neutrophils will show a more pronounced oxidative burst when exposed to ECFs from biofilm bacteria.
3. Removal of LPS will decrease neutrophil oxidative burst compared to samples with a full contingent of LPS.
4. Finally, we anticipate that polymyxin B (PMB) treatment will inactivate LPS and the neutrophils exposed to PMB-treated samples will respond in a similar fashion to neutrophils exposed to LPS-reduced samples.

1.7 Specific objectives

The specific objectives of this project are as follows:

1. Characterize the anaerobic mixed-species biofilms using viable cell counts, Confocal Scanning Laser Microscopy (CSLM) and Scanning Electron Microscopy (SEM).
2. Generate bacterial cell-free supernatants containing ECFs from mixed-species planktonic bacteria and biofilm bacteria and measure neutrophil oxidative responses to each.
3. Characterize the mixed-species ECFs by quantifying the amount of protein and LPS present in the growth media from planktonic bacteria and biofilm bacteria.

4. Remove the LPS from the mixed-species ECFs and measure neutrophil oxidative responses to both LPS-reduced planktonic samples and LPS-reduced biofilm samples.
5. Incubate the ECFs with polymyxin B (PMB) to inactivate the lipid A portion of LPS and measure neutrophil oxidative responses to PMB-treated planktonic and PMB-treated biofilm samples.

MATERIALS & METHODS

2.1 Bacteria

Fusobacterium necrophorum subsp. necrophorum from the American Type Culture Collection (ATCC, Manassas VA USA) 27852, isolated from a case of ovine foot rot and *Porphyromonas levii* ATTC 29147, isolated from the rumen of cattle were the two bacterial strains used in all experiments. The bacterial strains were stored in 10% glycerol at -80^{o}C and primary cultures were grown from frozen stock on fastidious anaerobic agar (FAA) (Acumedia, Michigan USA) supplemented with 5 μg/mL hemin (Sigma-Aldrich H9039), 0.2 μg/mL vitamin K_1 (Sigma-Aldrich V3501) and 5% (v/v) sterile defibrinated sheep blood (Dalynn Biologicals). Primary cultures were incubated for 48 hours under anaerobic conditions (5% hydrogen, 5% carbon dioxide and 90% nitrogen) in a Bactron II Anaerobic Chamber (Sheldon Manufacturing), then secondary sub-cultures were streak-plated and incubated anaerobically for 48 hours prior to use in experiments.

2.2 Mixed-species planktonic growth

Planktonic growth in a liquid medium was generated under anaerobic conditions using a modification of the direct colony suspension method (Ceri *et al.,* 1999). For each bacterium, three to five colonies were swabbed from a secondary sub-culture and suspended in 2 mL of brain-heart infusion broth (BHI; Becton, Dickinson and Company) supplemented with 5 μg/ml hemin and 0.2 μg/ml vitamin K_1. The suspensions were matched to a 1.0 McFarland standard and 500 μL of each standardized suspension was diluted in 14.5 mL BHI. To verify bacterial numbers, each inoculum was serially diluted, spot-plated with 20 μL spots on FAA and the number of colony-forming units (CFU) per

mL was determined. The two suspensions were mixed in a 1:1 ratio in a 50 mL polypropylene centrifuge tube (BD Falcon 352070) and incubated under anaerobic conditions for 18 hours. Gram stains were performed to ensure the media only contained the two bacteria of interest. The stains were observed using a light microscope at 100x magnification with an oil-immersion lens. With the differences in cellular morphology it was possible to identify each species (Figure 4).

2.3 Mixed-species biofilm growth

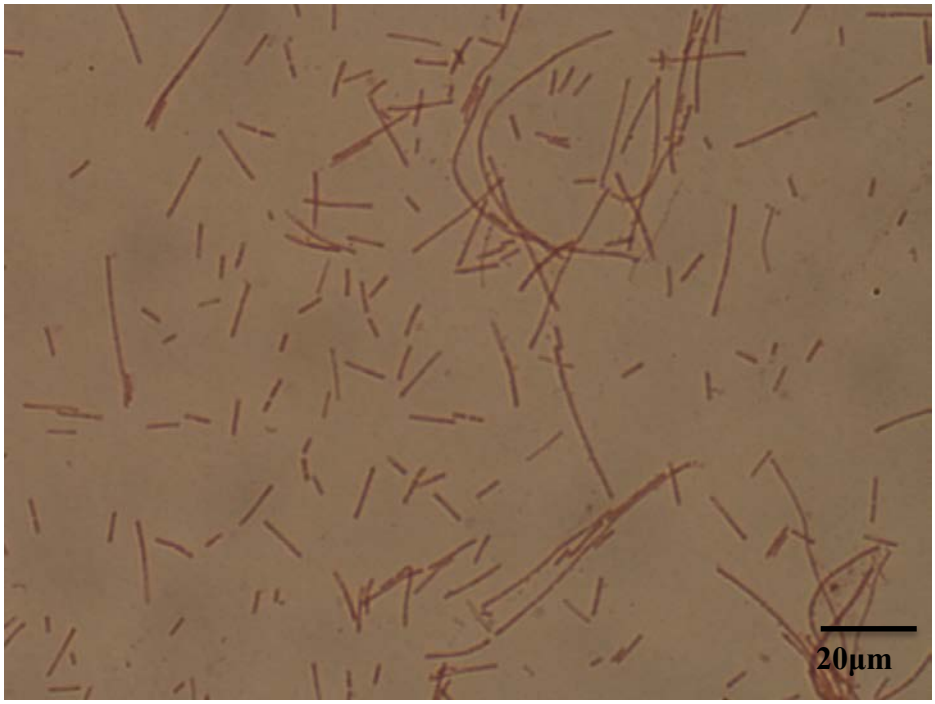
Bacterial suspensions in BHI were generated under anaerobic conditions following the procedure described above for planktonics. Once mixed in a 1:1 ratio, 1.5 mL of the mixed-species suspension was added to the wells of a 12-well culture plate (BD Falcon 353503). A Transwell insert with a 0.4 μm polycarbonate membrane (Corning 3401) was placed in each well and 500 μL of BHI was added on top (Figure 5). The plate was placed on a shaker (Scilogex MxM) at 115 Revolutions Per Minute (RPM) and incubated for 12 days. Every 24-48 hours, 500 μL of spent media was removed and 700 μL of new BHI was added to ensure fresh nutrients were supplied to bacteria in the biofilm present on the underside of the Transwell membrane.

To determine the number of viable cells present in the biofilms, the membranes were removed from the plate, placed in 1 mL of sterile 0.9% NaCl and sonicated for 5 min with an Aquasonic sonicator (VWR Scientific). The NaCl solution containing the cells from the biofilm was then serially diluted and spot-plated on FAA to determine CFU/mL. *P. levii* colonies exhibited a black pigment following incubation (>48 hours) and this was used as the criterion to differentiate from *F. necrophorum* colonies (Figure 6; Summanen *et al.,* 2005). Gram stains were performed to ensure the biofilms consisted

Figure 4. Cellular morphology of *F. necrophorum* and *P. levii*.

Gram stains were performed on secondary subcultures of *F. necrophorum* and *P. levii*. **A:** *F. necrophorum.* Long, pleomorphic rods are characteristic of the cellular morphology of this species. **B:** *P. levii.* Short rods or even cocci-shaped cells are typical of this species. Specimens were observed at 100x under oil-immersion.

A.



B.

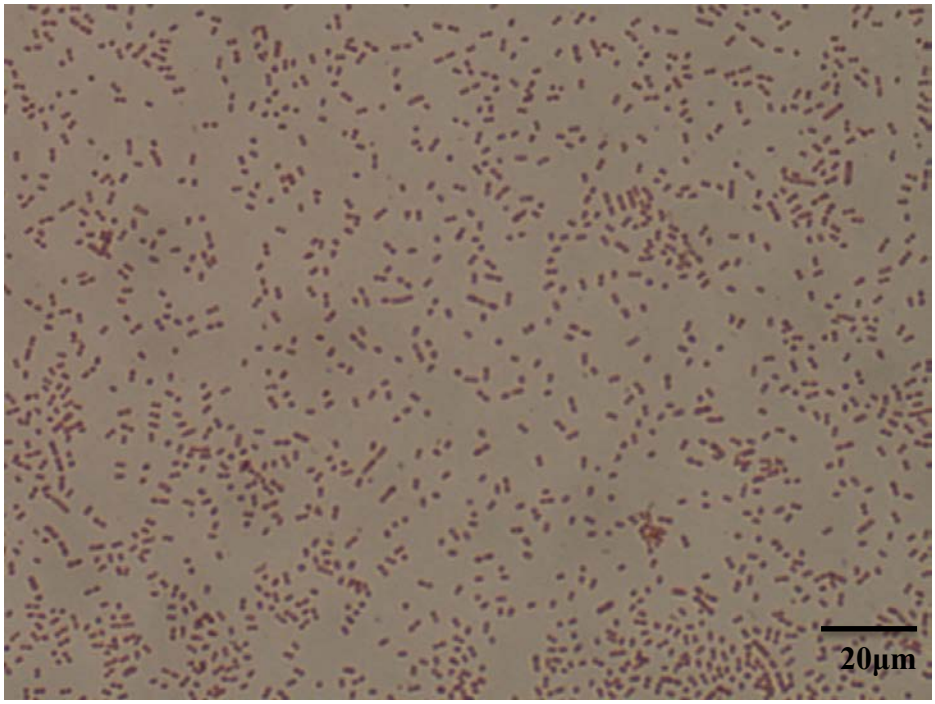


Figure 5. The Transwell system used to generate mixed-species biofilms

Diagram of the Transwell system used to grow biofilms. The upper compartment is separated from the lower compartment by a 0.4 μm polycarbonate membrane. 1.5 mL of standardized mixed-species inoculum was plated underneath the membrane with 0.5 mL BHI on top of the membrane.

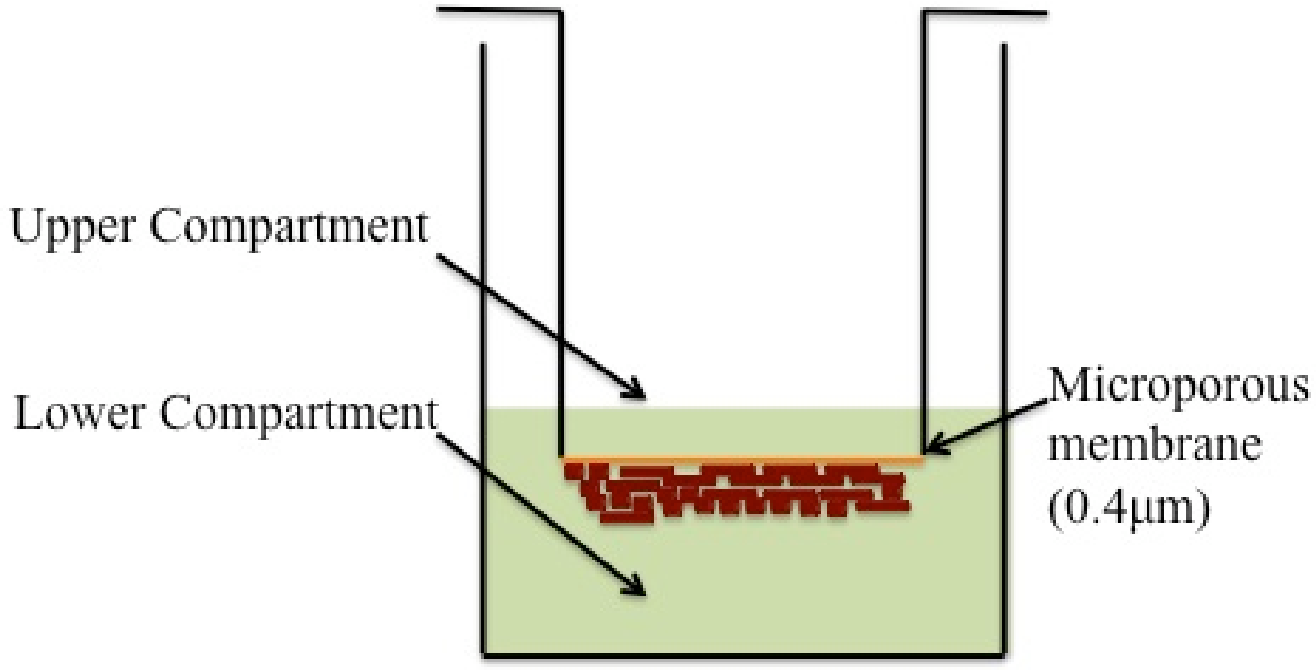
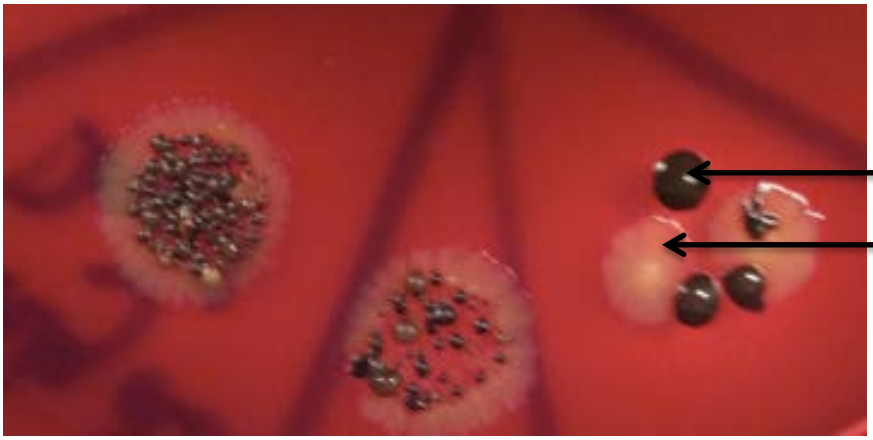

Upper Compartment
Lower Compartment
Microporous
membrane
(0.4μm)

Figure 6. Colony morphology of *F. necrophorum* and *P. levii.*

A mixed species culture grown for 72 hours on FAA supplemented with 5% defibrinated sheep blood. The culture was serially diluted, spot-plated and colonies were enumerated for a viable cell count. Colonies of *P. levii* were easy to differentiate from *F. necrophorum* due to the production of the black pigment (3 *P. levii* colonies on 10^{-5} dilution, 2 *F. necrophorum* colonies on 10^{-5} dilution). One colony of each species is indicated with an arrow.


P. levii
F. necrophorum

of the two bacteria of interest in an approximate 1:1 ratio.

To investigate the extent of biofilm growth, confocal scanning laser microscopy (CSLM) was used to physically characterize the biofilm. Briefly, membranes were rinsed in 0.9% NaCl to remove any planktonic cells then stained for 1 hour in 0.05% Acridine Orange (AO) (Sigma-Aldrich 318337) in distilled water. Images were taken at excitation wavelength 480 nm and emission 500 nm under a 40x water-immersion lens. A stack of 20 images was compiled and the distance the lens moved in the Z-direction was used to determine the depth of the biofilm. The images were taken with a Leica TCS SP2 confocal scanning microscope and data were analyzed with Leica Lite LCS software.

Scanning electron microscopy (SEM) was performed to ensure biofilm uniformity and confirm the morphotypes of the two species of interest in the biofilm. Membranes were washed in 2 mL of 0.9% NaCl to remove loosely-adherent planktonic bacteria and then fixed for 3 hours in 2 mL cacodylate buffer (0.1M) (Sigma C0125) containing 2.5% glutaraldehyde (Electron Microscopy Sciences 16365). The membranes were air-dried for 5 days at room temperature in a sterile 12-well culture dish and then mounted and sputter coated with gold-paladium. The biofilms were observed with a Phillips XL30ESEM microscope at an accelerating voltage of 20 kV and photographs were taken at magnifications of 100x, 1,000x and 10,000x.

2.4 Polymorphonuclear neutrophil purification

Whole blood was aseptically collected from healthy blood donor cattle (crossbred Angus heifers) via jugular venipuncture into vacutainers containing 1.5 mL acid citrate dextrose solution (BD Vacutainers 364606). The vacutainers were inverted several times to ensure the blood and anticoagulant were mixed prior to the 30-minute transportation

on ice back to the laboratory. Neutrophils were isolated using a repeated hypotonic lysis procedure previously described (Lee *et al.,* 2004). All procedures were performed at 4ºC or solutions were kept on ice. Briefly, blood was pooled into 50 mL polypropylene centrifuge tubes and centrifuged at 1200 x *g* for 20 minutes in an Allegra 6R desktop centrifuge (Beckman Coulter, Inc) without braking. The plasma and buffy coat were removed and the remaining cells were gently washed with 20 mL of sterile Hank's Balanced Salt Solution (HBSS; with $NaHCO_3$, without phenol red, calcium chloride or magnesium sulphate; Sigma-Aldrich H6648). The cells were centrifuged at 1200 x g for 10 minutes without braking and the HBSS along with any residual buffy coat was removed. Erythrocytes were eliminated by adding 20 mL of cold filter-sterilized hypotonic lysis solution (10.6 mM Na_2HPO_4, 2.7mM NaH_2PO_4; Sigma-Aldrich) for 1 minute, followed by the addition of 10 mL of cold filter-sterilized hypertonic restoring solution (10.6 mM Na_2HPO_4, 2.7mM NaH_2PO_4, 462mM NaCl). The tubes were gently inverted and the cell mixture was centrifuged at 1000 x g for 10 minutes without braking. The supernatant was discarded, and then the lysis procedure was repeated 2 additional times to ensure elimination of the erythrocytes. Following the third lysis step, the neutrophils were re-suspended in 10 mL of HBSS. The neutrophils were diluted ten-fold by placing 10 µL of cells in 40 µL HBSS and 50 µL of Trypan blue (Sigma-Aldrich T8154). Neutrophils were enumerated on a hemacytometer by counting all 4 large squares of the device and multiplying by the dimensions of the well. Viability was determined by the number of cells that excluded the Trypan blue stain and purity was assessed by performing differential cell counts on cytospin preparations (CytoSpin 4 Cytocentrifuge, Thermo Scientific) stained with Diff-Quik stain (Dade Behring B4132).

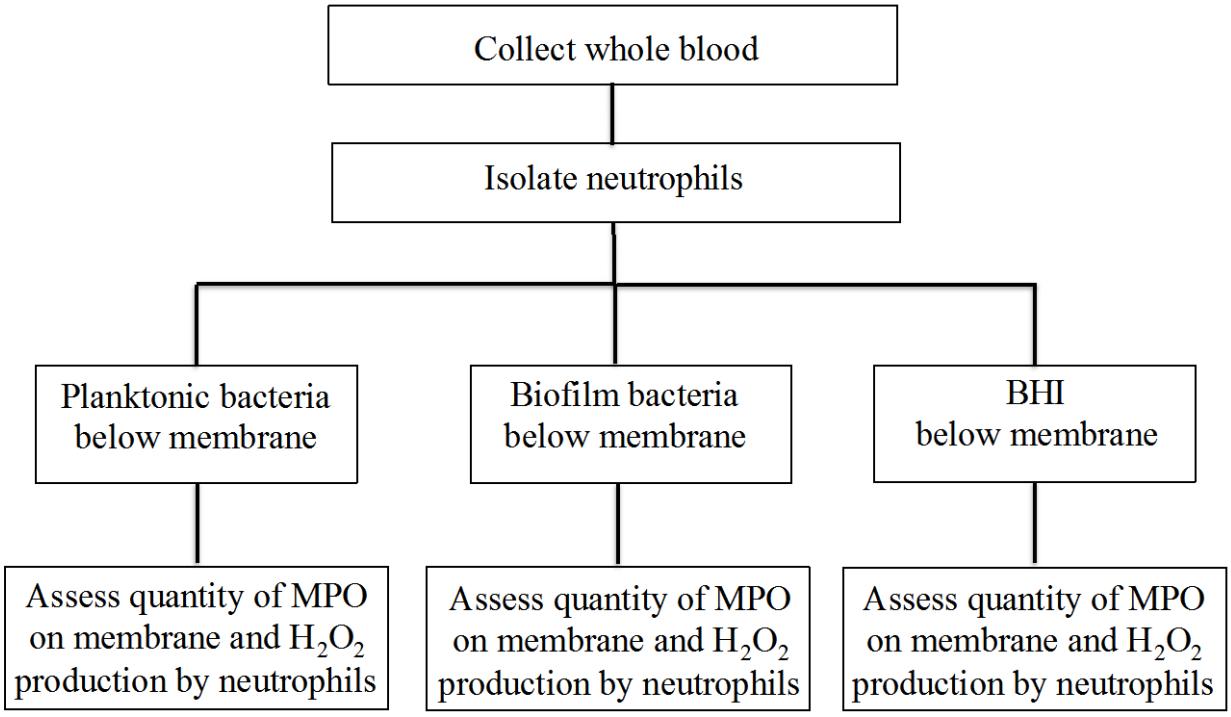
Neutrophil populations used for all experiments were greater than 90% pure and greater than 90% viable. Following enumeration, the neutrophil suspensions were diluted in HBSS to achieve the desired cell concentration for the various experiments. The first experiments were conducted in the Transwell system to assess the neutrophil responses to planktonic bacteria, biofilm bacteria or the control BHI media across the polycarbonate membrane (Figure 7).

2.5 Quantification of neutrophils on the Transwell membrane

A myeloperoxidase (MPO) assay was developed, including modifications to the manufacturers' instructions for determining the quantity of neutrophils that were present on the Transwell membranes with planktonic bacteria, biofilm bacteria or BHI media underneath (Fluoro MPO Detection Kit; Cell Technology). MPO is an enzyme present in the cytoplasmic granules of neutrophils that uses hydrogen peroxide to generate a wide variety of bactericidal compounds (DeLeo *et al.*, 1999). MPO is often employed as an indicator of neutrophil quantity (Hampton *et al.,* 1998). The assay uses a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce a fluorescent analog. For this experiment, the biofilms on the Transwell membrane were removed from their original cassette with a sterile scalpel and then placed under a fresh membrane in 1.7 mL BHI. In a 12-well culture plate, 500 µL of purified neutrophils ($5x10^6$ cells/mL) were placed on top of Transwell membranes with 1.7 mL of planktonic bacteria, biofilm bacteria or BHI media on the opposite side of the membrane (lower compartment) (Figure 5). The ratio of neutrophils to bacteria was approximately 1:10 to standardize assays (Mills *et al*., 2006; Permpanich *et al.,* 2006).

Figure 7. Experimental design for studies that investigated neutrophil responses across the Transwell membrane.

Flow chart that shows the general outline of the experiments that investigated the oxidative responses of neutrophils that were indirectly exposed to planktonic bacteria, biofilm bacteria or BHI control media across the Transwell membrane.


Collect whole blood
Isolate neutrophils
Planktonic bacteria below membrane
Biofilm bacteria below membrane
BHI below membrane
Assess quantity of MPO on membrane and H_2O_2 production by neutrophils
Assess quantity of MPO on membrane and H_2O_2 production by neutrophils
Assess quantity of MPO on membrane and H_2O_2 production by neutrophils

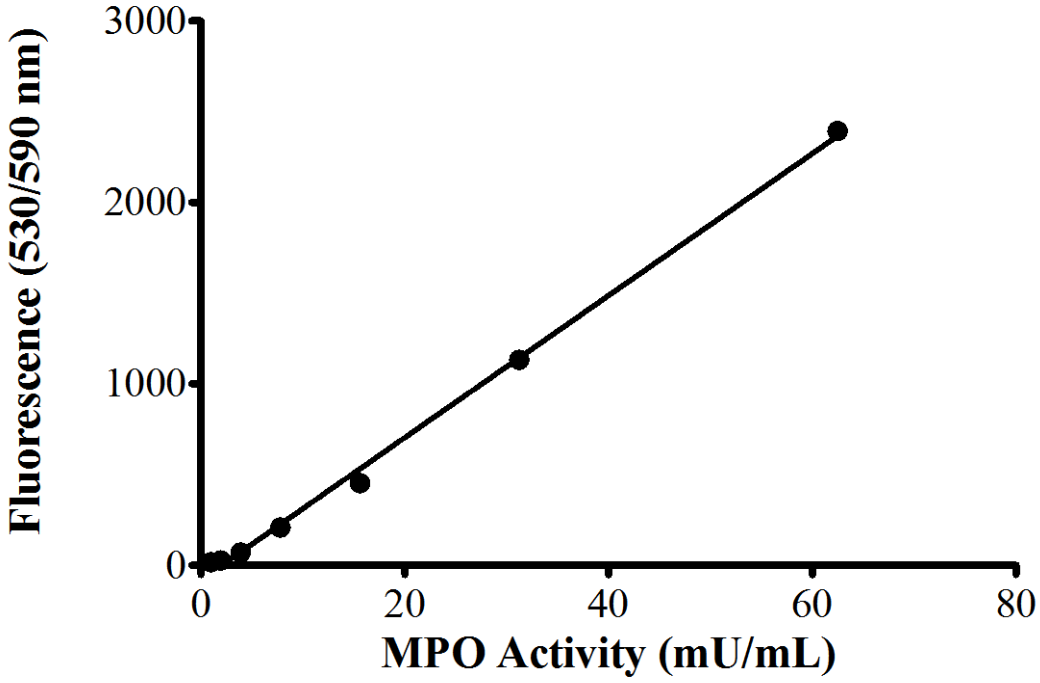
The plate was incubated for 60 minutes at 37ºC and 5% CO_2, then the fluid in the upper chamber of the Transwell was discarded. The membranes that separated the neutrophils from the various stimuli were removed from the Transwell cassette with a scalpel and placed in the wells of a new 12-well culture plate each containing 1.0 mL of ice-cold 0.5% hexadecyltrimethylammonium bromide (HTA-Br) (Sigma-Aldrich H9151). Neutrophils that were present on the membrane were lysed by gently shaking the plate at 100 RPM for 20 minutes at 4ºC on a horizontal shaker and then the lysate was diluted 1/167 with assay buffer (Cell Technology FLMPO 100-3). Fifty microliters of lysate was added in triplicate to the wells of a black plate (Grenier BioOne 655090). The plate also contained 8 triplicate wells each with 50 µL of diluted MPO (from 0 to 62.5 mU/mL) for the standard curve, which was performed for each experiment (Figure 8). Immediately before being added to the samples or standards in the black plate, 5 mL of MPO reaction cocktail was generated by mixing 50 µL of Detection Reagent with 5 µL of hydrogen peroxide in 4.945 mL of assay buffer (Cell Technology). Fifty microliters of reaction cocktail was added to each well and the plate was incubated in the dark at room temperature for 45 minutes. Raw fluorescence was read at excitation wavelength 530 nm and emission 590 nm in a fluorescence plate reader (Molecular Devices; Fisher Scientific) and MPO activity on the membrane was determined using the standard curve generated from the known concentrations of MPO stock.

2.6 Assessment of neutrophil oxidative activity in the top chamber of Transwells

The amount of hydrogen peroxide produced by the neutrophils in the upper chamber was used as a measure of cell oxidative activity. Hydrogen peroxide production

Figure 8. Standard curve for MPO activity.

MPO was diluted in water to generate a standard curve of MPO activity. Fluorescence was measured at excitation wavelength 530 nm and emission wavelength 590 nm. The standard curve was generated for each independent experiment. R^2=0.99743.


Fluorescence (530/590 nm)
3000
2000
1000
0
0
20
40
60
80
MPO Activity (mU/mL)

was assessed with the 2'7'dichlorodihydrofluorescein diacetate (DCF-DA) assay. DCF is a fluorimetric detector of hydrogen peroxide and can be used to measure intracellular oxidative burst (Krejsa and Schieven, 2000). The assay is based on the following reaction:

$$H_2O_2 + 2H_2DCF \rightarrow 2H_2O + 2DCF \text{ (Fluorescent product)}$$

Oxidation of DCF by hydrogen peroxide releases the fluorescent product that can be quantified in a fluorescence plate reader at excitation 485 nm and emission 530 nm (De Leo *et al.,* 1999). DCF-DA (Sigma-Aldrich D6883) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich D8418) at a concentration of 5 mM, aliquoted and stored in the dark at -20ºC until use in experiments (Wang and Joseph, 1999; Wang *et al.,* 2008; Rastogi *et al.,* 2010). Neutrophils ($8x10^6$ cells/mL) were isolated as described above and re-suspended in 25 μM HBSS/DCF (50 μL of stock DCF in 9.950 mL HBSS). The neutrophils were incubated at 37ºC + 5% CO_2 for 25 minutes and then 500 μL of the cells were placed in the upper chamber of a Transwell with 1.7 mL of planktonic bacteria, biofilm bacteria or BHI in the lower compartment on the opposite side of the membrane (indirect exposure). The plate was incubated in the dark at 37ºC + 5% CO_2 for 1 hour, then the neutrophil suspensions from each of the Transwells were removed to a black microplate (100 μL/well in triplicate). The black plate was incubated in the dark at 37ºC and 5% CO_2 for 3 hours. The fluorescence was measured at 485 nm and 530 nm every 30 minutes for the duration of the incubation. Raw fluorescence was used as an indication of overall oxidative burst.

2.7 Isolation of extracellular factors in bacterial growth media

Mixed species planktonic bacteria and biofilm bacteria were prepared in BHI media as described above in sections 2.2-2.3 and then the growth media from each was placed into 15 mL centrifuge tubes (Falcon 352095). The tubes containing growth media from planktonic bacteria or biofilm bacteria were centrifuged at 3200 x *g* at 4ºC to pellet the bacteria and then the media was passed through a 0.2 μm filter (Nalgene 596-4520) to remove any remaining bacteria. Anaerobic and aerobic controls were plated on FAA to ensure all live bacteria were removed from the growth media. The samples containing the ECFs from planktonic bacteria or biofilm bacteria were aliquoted into 1 mL aliquots and stored at -80ºC until use in experiments. Neutrophils were exposed directly to the growth medium containing ECFs from planktonic bacteria or biofilm bacteria for the remaining experiments (Figure 9).

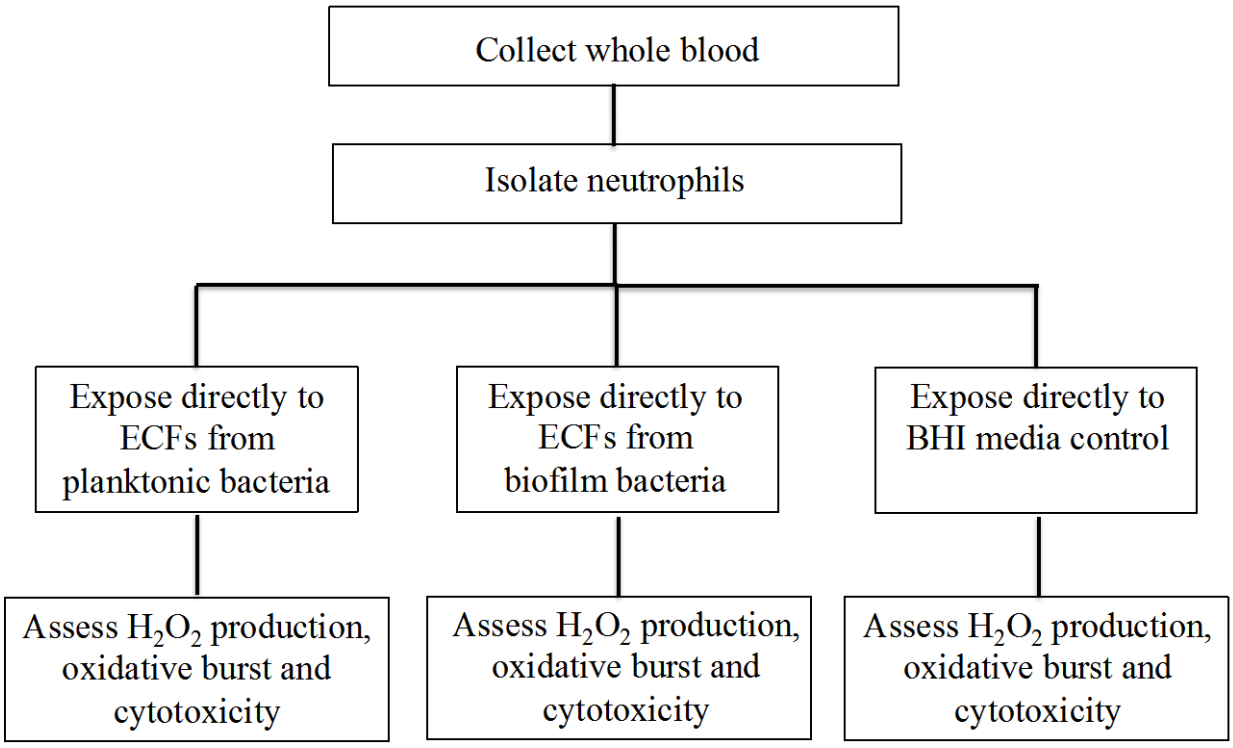
2.8 Neutrophil responses to extracellular factors in growth media

2.8.1 Dichlorodihydrofluorescein diacetate assay

The DCF-DA assay was employed as a measure of neutrophil activation following direct exposure to the ECFs from planktonic bacteria, biofilm bacteria or BHI. Twenty microliters of 5 mM stock DCF-DA was diluted in 9.980 mL HBSS to generate a 10 μM working solution of HBSS/DCF immediately prior to the experiment. Neutrophils ($6.0x10^6$cells/mL) were isolated as described above and were re-suspended in 5 mL of HBSS/DCF. The cells were incubated at 37ºC and 5% CO_2 for 25 minutes to allow the DCF-DA to enter the cells. During this incubation, the extracellular factors from planktonic bacteria, biofilm bacteria or BHI were placed in 6 wells of a 96-well black plate (150 μL/well). Fifty microliters of neutrophils in HBSS/DCF was added to 3 of the

Figure 9. Experimental design for studies that investigated the responses of neutrophils that were directly exposed to the ECFs from planktonic bacteria, biofilm bacteria or the BHI media control

Flow chart that shows the general outline of the experiments that investigated the oxidative responses of neutrophils that were directly exposed to the growth media containing ECFs from planktonic bacteria, biofilm bacteria or BHI.


Collect whole blood
Isolate neutrophils
Expose directly to ECFs from planktonic bacteria
Expose directly to ECFs from biofilm bacteria
Expose directly to BHI media control
Assess H_2O_2 production, oxidative burst and cytotoxicity
Assess H_2O_2 production, oxidative burst and cytotoxicity
Assess H_2O_2 production, oxidative burst and cytotoxicity

wells with extracellular factors and 50 µL of HBSS/DCF (without neutrophils) was added to the other 3 wells containing the extracellular factors. These neutrophil-free controls accounted for the background present in the samples. The black plate was incubated in the dark at 37°C and 5% CO_2 for 2 hours. The fluorescence was measured at 485 nm and 530 nm every 20 minutes for the duration of the incubation. Raw fluorescence was used as an indication of overall oxidative burst.

2.8.2 Nitroblue tetrazolium assay

To assess overall oxidative burst and reinforce the data obtained in the DCF assay, a nitroblue tetrazolium (NBT) assay was conducted. Neutrophil oxidative burst was quantified by measuring the reduction of NBT to formazan, which produces a dark blue pigment and absorbance can be measured in a spectrophotometer at 620 nm (Choi *et al.*, 2006). The NBT substrate solution was prepared by dissolving a 10 mg tablet of NBT (Sigma-Aldrich N5514) in 10 mL of HBSS to make a 1 mg/mL solution (HBSS/NBT). The NBT solution was vortexed and then passed through a 0.45 µm syringe filter to remove any remaining undissolved NBT particles. Purified neutrophils ($7.0x10^6$cells/mL) were re-suspended in 5 mL of HBSS/NBT. The cells were then directly exposed to 900 µL of the ECFs from planktonic bacteria, biofilm bacteria or BHI and incubated at 37°C and 5% CO_2 for 1 hour. Following treatment, neutrophils were washed three times with HBSS to remove any excess NBT reagent. Conversion of NBT to formazan was determined using a colorimetric method developed previously (Choi *et al.,* 2006; Wu *et al.,* 1999). Briefly, formazan deposits were solubilized by the addition of 120 µL of 2M KOH and 140 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich D8418). The mixture was mixed on a horizontal shaker for 10 minutes and then 100 µL of the solutions were

transferred to the wells of a black 96-well plate. The absorbance was measured at 620 nm using a SpectraMAX M2e microplate reader (Molecular Devices). The absorbance values were measured in triplicate and reported as an overall indication of oxidative burst.

2.8.3 Neutrophil viability (Cytotoxic effects of extracellular factors)

In addition to viable counts performed using Trypan blue staining and a hemacytometer, a lactate dehydrogenase (LDH) assay was employed to investigate the cytotoxic effects of bacterial extracellular factors on the neutrophils. LDH is an intracellular enzyme present in neutrophils that catalyzes the conversion of lactate to pyruvate and can be used as an indicator of cellular lysis (Korzeniewski and Callewaert, 1983; Decker and Lohmain-Matthes, 1988). The concentration of LDH in the cellular supernatant was determined using a commercially available kit (Roche Applied Science 11 644 793 001). LDH present in the supernatant reduces NAD^+ to $NADH^+$ by oxidation of lactate to pyruvate and this reaction is coupled to the reduction of tetrazolium salt to formazan. The amount of formazan that is produced can be detected with a spectrophotometer by measuring absorbance at 492 nm, which is proportional to the number of lysed cells (Roche instruction manual). The assay was carried out according to the manufacturers' instructions with a few modifications. Neutrophils ($6x10^6$cells/mL) were exposed to the extracellular factors from planktonic bacteria, biofilm bacteria or BHI media for 1 hour at 37^{o}C and 5% CO_2. Following the incubation, the solutions were centrifuged at 1200 x g to remove the neutrophils and the supernatant was analyzed for LDH activity. Cytotoxicity was represented as a proportion of total LDH release, as determined by treatment with 2.5% Triton X-100 (Sigma-Aldrich X100) for 1 hour at 37^{o}C and 5% CO_2 as a positive control.

2.9 Characterization of the extracellular factors from growth media

2.9.1 Protein concentration

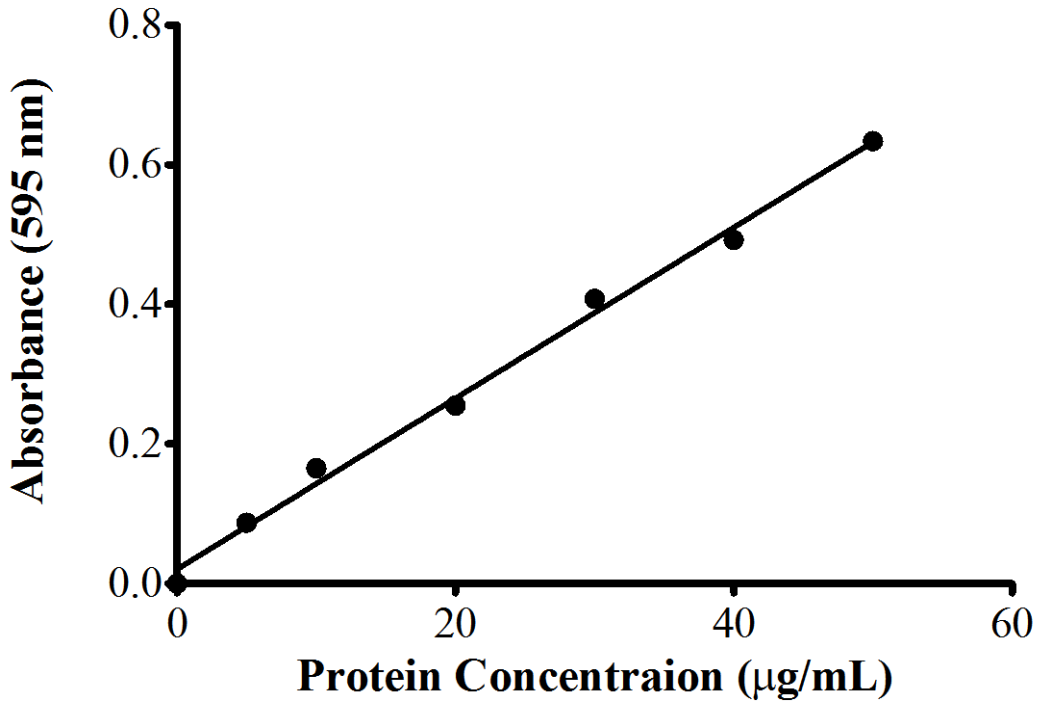
The amount of protein present in the ECFs from planktonic bacteria, biofilm bacteria or control BHI was quantified using the Bio-Rad microtiter protein assay. A standard curve was generated from bovine serum albumin (BSA; Sigma-Aldrich A-2153), using dilutions of BSA in water from 5 μg/mL to 50 μg/mL (Figure 10). 160 μL of each sample or standard was pipetted into triplicate wells of a black 96-well plate. Forty microliters of Bio-Rad dye reagent concentrate (Bio-Rad 500-006) was added to each well using a multi-channel pipet and mixed by pipetting up and down. The plate was covered and incubated at room temperature for 15 minutes. Absorbance was read in a microplate reader at 595 nm and the protein concentrations of the extracellular factors from planktonic bacteria, biofilm bacteria and BHI were determined using the standard curve (Figure 10).

2.9.2 LPS concentration

To determine the amount of LPS present in the growth media containing ECFs from planktonic bacteria and biofilm bacteria, a commercially available Limulus Ameobocyte Lysate (LAL) assay was used (Pierce Thermo Scientific 88282). Factor C (an enzyme present in the lysate from horseshoe crabs) is activated in the presence of endotoxin. Once activated, the enzyme catalyzes the splitting of *p*-Nitroaniline (pNA) from the colourless substrate. The colour development can be measured at 405 nm and the intensity is proportional to the amount of LPS present in the sample. A standard curve was generated from dilutions of a known concentration of *Escherichia coli* (*E. coli*) endotoxin (in endotoxin units per mL (EU/mL)) and used to calculate the total amount of

Figure 10. Standard curve for protein concentration.

Bovine serum albumin was diluted in water to generate a standard curve. The absorbance was measured at 595 nm. R^2 =0.99432.


0.8
0.6
0.4
0.2
0.0
Absorbance (595 nm)
0
20
40
60
Protein Concentraion (μg/mL)

LPS present in the growth media containing ECFs from planktonic bacteria and biofilm bacteria (Figure 11). The assay was carried out according to a previously described protocol (Borzecka et al., 2013). Briefly, a black microplate was equilibrated in a heating block for 10 minutes at 37^{o}C. The plate was maintained at 37^{o}C while 50 µL of each standard or diluted unknown samples were added to the wells (in triplicate). The plate was incubated at 37^{o}C for 5 minutes to ensure that the temperatures were all equivalent. Fifty microliters of the LAL reagent was then added to each well using a multi-channel pipet and mixed with the samples by pipetting up and down several times. After exactly 10 minutes, 100 µL of the substrate solution was added to all wells. Special care was taken to ensure proper mixing and a consistent pipetting speed. The plate was incubated in the dark for an additional 6 minutes and then 50 µL of 25% (v/v) acetic acid was added to stop the reaction. The optical density was measured at 405 nm and a standard curve from 0.1 EU/mL to 1 EU/ml was used to calculate the concentration of LPS in the extracellular factors from planktonic bacteria and biofilm bacteria.

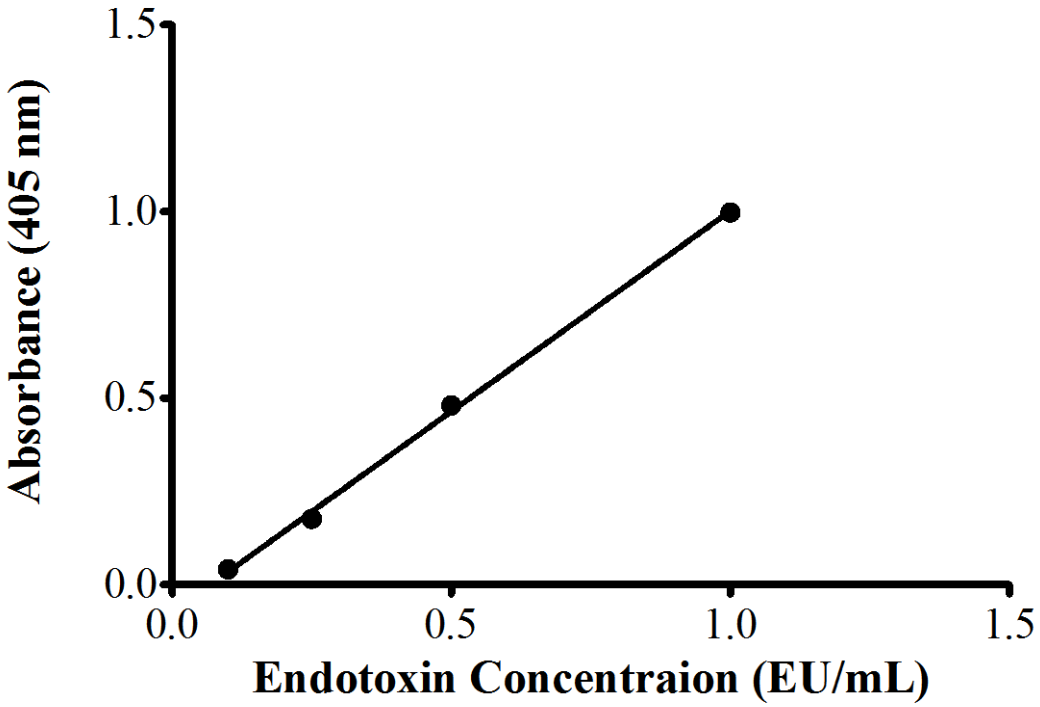
2.10 LPS removal

2.10.1 Endotoxin-removal resin

LPS was removed from the growth media containing ECFs from planktonic bacteria or biofilm bacteria using columns containing a special endotoxin-removal resin (Pierce Thermo Scientific 88274). The resin contains beads with modified poly-L-lysine attached, which has a high affinity for LPS and can reduce endotoxin levels by up to 99%. The assay was carried out according to the manufacturers' protocol. Briefly, 6 of the columns were regenerated by incubating the resin with 3.5 mL of 0.2 N NaOH at room temperature overnight with gentle shaking. Two columns were then placed in a 15

Figure 11. Standard curve for endotoxin concentration.

A standard curve was prepared using LPS from *E. coli* diluted in water. The absorbance was measured at 405 nm. The standard curve was generated for each independent experiment. R^2=0.99869.


1.5
1.0
0.5
0.0
Absorbance (405 nm)
0.0
0.5
1.0
1.5
Endotoxin Concentraion (EU/mL)

mL collection tube and centrifuged at 500 x g to remove the solution. Next, 3.5 mL of 2M NaCl was mixed with the resin and then the tubes were centrifuged again to remove the salt solution. The resin was washed with 3.5 mL of endotoxin-free H_2O, followed by three repeated washes with 3.5 mL of endotoxin free buffer (Tris-HCl). The growth media containing ECFs from planktonic bacteria or biofilm bacteria was added to the two columns. The columns were placed on a rocker for 1 hour at room temperature. During the incubation, 2 new columns were prepared using the same protocol described above. After 1 hour, the columns containing the ECFs were placed in collection tubes and centrifuged at 500 x g for 1 minute to collect the LPS-reduced fractions from planktonic bacteria or biofilm bacteria. The endotoxin removal process was repeated two more times to ensure complete removal of the LPS from the ECFs from planktonic bacteria or biofilm bacteria. Following the final incubation with the removal-resin, the samples were aliquoted into 1 mL aliquots and stored at -80^{o}C until use in experiments. The amount of LPS in these column-treated samples was quantified with the LAL assay and then they were used in neutrophil exposure experiments to investigate neutrophil responses to LPS-reduced planktonic or biofilm ECFs.

2.10.2 Polymyxin B

Polymyxin B (PMB) is a cyclic, cationic antibiotic that is known to act on LPS. It binds to the gram-negative cell wall, making it more permeable and the resulting influx of water leads to bacterial cell death (Cooperstock, 1974). PMB has been demonstrated to be efficient at inactivating endotoxins in solution by binding to the lipid A component of LPS and this phenomenon was used to reinforce the results of the endotoxin-removal resin (Morrison and Jacobs, 1976). The concentration of PMB and incubation times had

to be optimized for this experiment. ECFs from planktonic bacteria and biofilm bacteria were prepared as described in Section 2.6. A range of concentrations (1, 5, and 10 mg/mL) of PMB were added to the media from planktonic bacteria, biofilm bacteria or BHI and the tubes were incubated for 1 hour at 37^{o}C and 5% CO_2 to allow time for the antibiotic to bind the LPS present in each sample. The PMB-treated ECFs from planktonic bacteria, biofilm bacteria or control BHI were used in neutrophil exposure experiments to investigate neutrophil responses to LPS-reduced planktonic or biofilm ECFs.

2.11 Statistical analyses

All values were expressed as mean +/- standard error of the mean (SEM). Statistical analyses were performed using Prism 5 software. All experiments with normally distributed data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test, in order to compare specific groups. Statistical significance was established at $p < 0.05$ using multiple replicates of a minimum of 3 separate, independent experiments.

Results that were not normally distributed, as determined by the D'Agostino and Pearson normality test, were analyzed non-parametrically using a Kruskal-Wallis one-way analysis of variance followed by a Dunn's test to compare specific groups. Statistical significance was established at $p < 0.05$ using multiple replicates of a minimum of 3 separate, independent experiments.

RESULTS

3.1 Biofilm characterization

3.1.1 Viable cell counts

Viable cell counts were performed to determine the quantity of bacteria present in planktonic cultures and the quantity of bacteria growing on the membrane as a biofilm. The results demonstrate that after 12 days of growth in the Transwell system, the ratio of *F. necrophorum* to *P. levii* remained approximately 1:1 (Figure 12).

3.1.2 Confocal microscopy

Confocal scanning laser microscopy (CSLM) was used to measure the depth of the biofilms present on the Transwell membranes. Following a brief wash step to remove any planktonic bacteria, all bacteria present on the membrane were stained with the general bacterial stain AO. The results indicate that the biofilms are uniformly distributed across the membrane (Figure 13A). The average depth of the biofilms was approximately 60 μm, with a stained sterile membrane acting as the negative control (Figure 13B). The stained sterile membrane showed no fluorescence (data not shown).

3.1.3 Scanning electron microscopy

SEM was employed to verify that the biofilms consisted of the two bacterial species of interest (*F. necrophorum* and *P. levii*). One sterile membrane was imaged as a negative control (Figure 14A). At a high magnification (10,000x) it is evident that the biofilms are uniformly distributed and consist of the bacterial morphotypes consistent with *F. necrophorum* and *P. levii* (Figure 14B).

Figure 12. Viable cell counts (CFU/mL) of planktonic cultures and biofilms.

Planktonic cultures were grown overnight in BHI. Biofilms were grown on Transwell membranes for 12 days with BHI media changes every 48 hours. Membranes were washed 3 times in 0.9% NaCl to remove any planktonics and then sonicated into 0.9% NaCl. Cultures were serially diluted in 0.9% NaCl and spot-plated on FAA to enumerate viable cells. Counts were performed 3-4 days after plating to allow *P. levii* colonies to develop pigment for differentiation from *F. necrophorum*. n= 13.

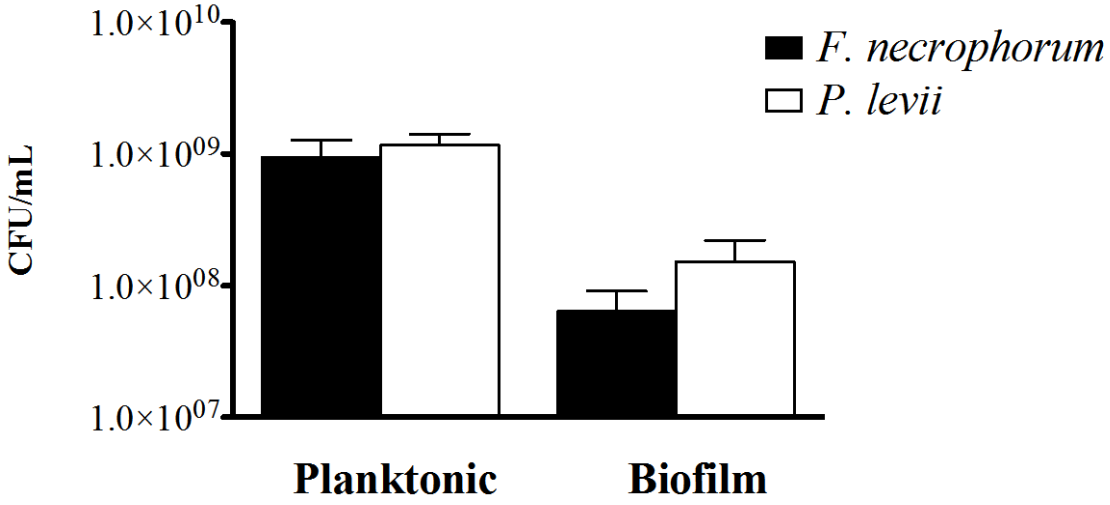
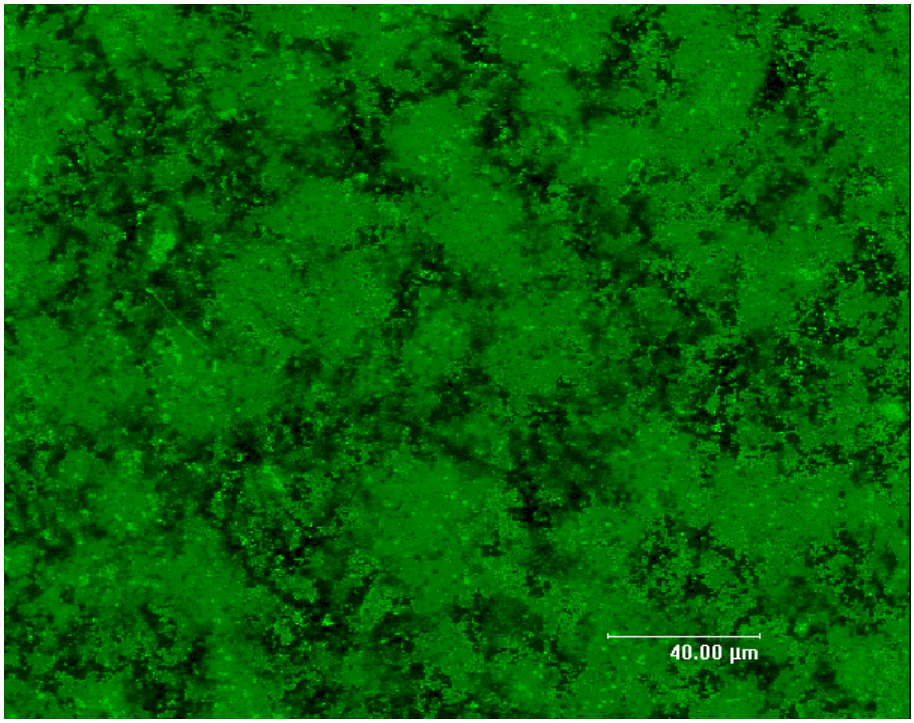

1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
CFU/mL
F. necrophorum
P. levii
Planktonic
Biofilm

Figure 13. Confocal microscopy on mixed-species biofilms.

Biofilms were grown on Transwell membranes for 12 days with BHI media changes every 48 hours. Membranes were washed 3 times in 0.9% NaCl to remove any planktonics and then stained with 1% Acridine Orange (AO) for 1 hour at room temperature. Images were taken at excitation wavelength 480 nm and emission 500 nm. **A:** overlay of images taken, **B:** 3-dimensional view showing the depth of the biofilm.

A.


40.00 µm

B.

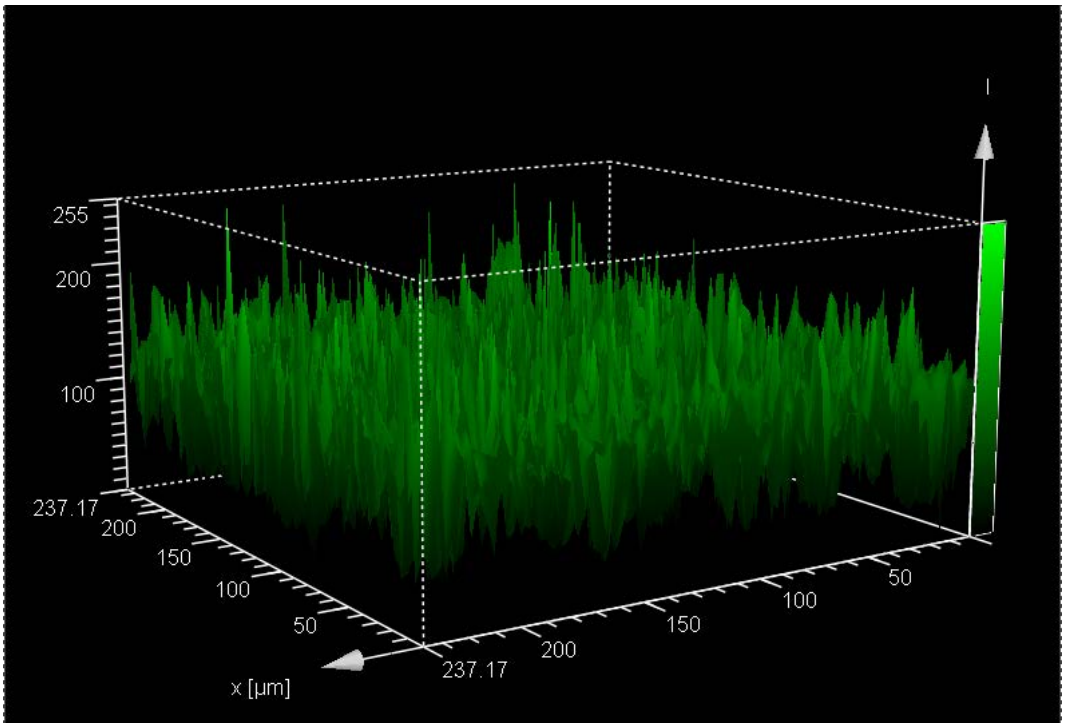
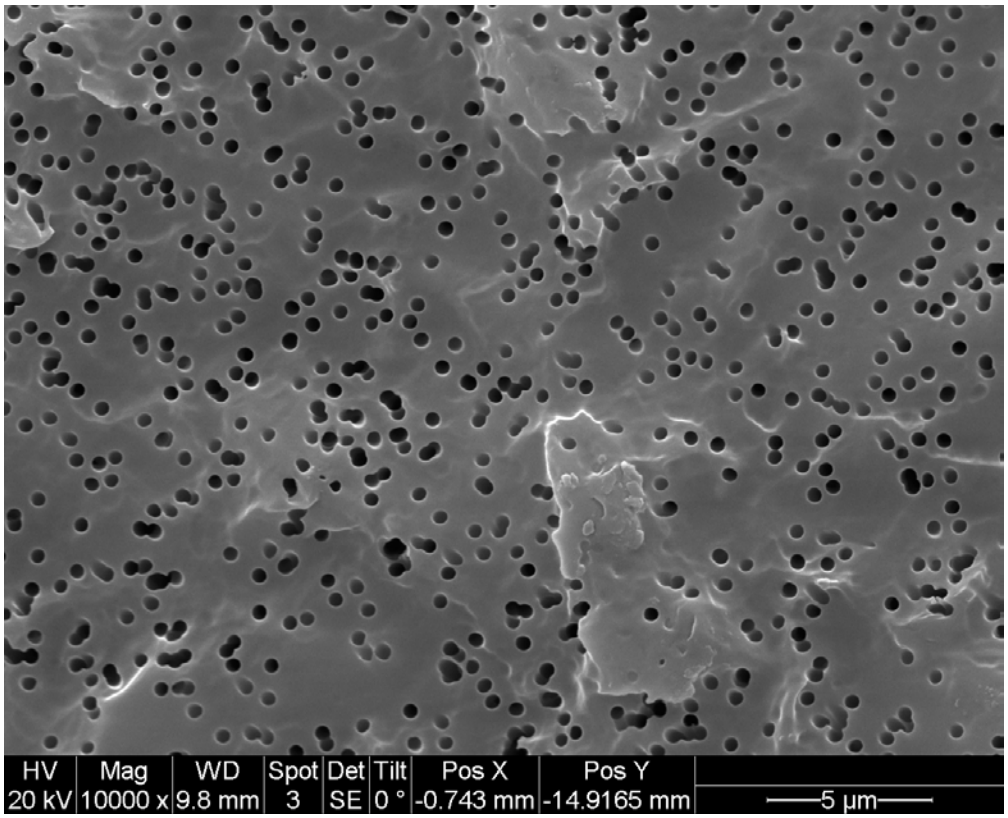

I
255
200
100
237.17
200
150
100
50
x [µm]
237.17
200
150
100
50

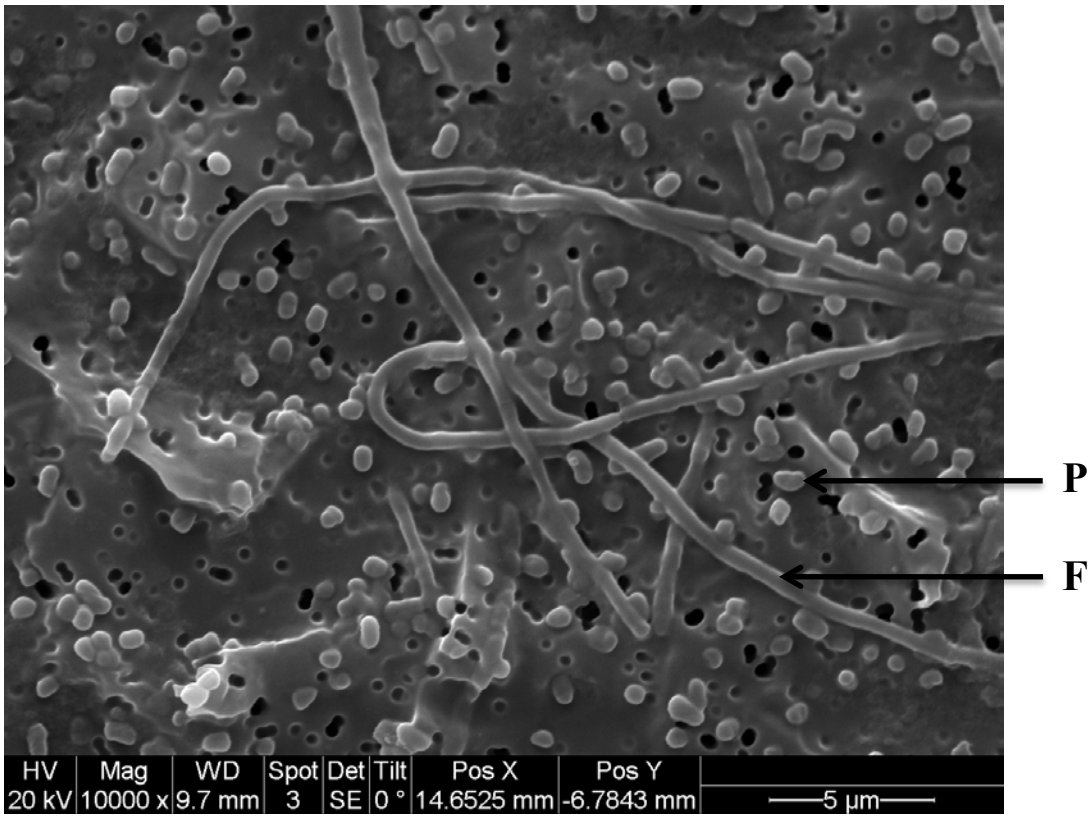
Figure 14. Scanning electron microscopy of mixed-species biofilms.

Biofilms were grown on Transwell membranes for 12 days with BHI media changes every 48 hours. Membranes were washed 3 times in 0.9% NaCl to remove any planktonics and fixed for 3 hours in 2 mL cacodylate buffer containing 2.5% glutaraldehyde then air-dried for five days prior to imaging. *F. necrophorum* (**F**) forms the long rods and *P. levii* (**P**) are oval-shaped (indicated with arrows). **A:** Sterile membrane. **B:** 1:1 Biofilm growth. Magnification 10,000x.

A.


HV
20 kV
Mag
10000 x
WD
9.8 mm
Spot
3
Det
SE
Tilt
0 °
Pos X
-0.743 mm
Pos Y
-14.9165 mm
5 µm

B.


P
F
HV
20 kV
Mag
10000 x
WD
9.7 mm
Spot
3
Det
SE
Tilt
0 °
Pos X
14.6525 mm
Pos Y
-6.7843 mm
5 µm

3.2 The quantity of neutrophils present on Transwell membranes does not vary regardless of the sample in the lower compartment

The number of neutrophils present on Transwell membranes when neutrophils were incubated for 1 hour at 37^{o}C + 5% CO_2 in the upper compartment with planktonic bacteria, biofilm bacteria or BHI underneath was determined by measuring MPO activity on the membranes. MPO activity was assessed by measuring fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. As shown in Figure 15, there were no significant differences in the quantity of neutrophils present on the membranes with planktonic bacteria, biofilm bacteria or BHI underneath.

3.3 Neutrophils exhibit different levels of oxidative activity when indirectly exposed to planktonic bacteria, biofilm bacteria or BHI across the Transwell membrane

The MPO assay demonstrated that the same number of neutrophils was present in each Transwell and the next experiment was carried out to investigate the oxidative burst of these neutrophils by measuring hydrogen peroxide production using the DCF assay. Fluorescence at excitation wavelength 485 nm and emission 530 nm was used to assess hydrogen peroxide production, which corresponds to overall oxidative burst. The results of these experiments demonstrated that neutrophils exposed to planktonic bacteria across the membrane exhibit a significantly higher level of oxidative activity than neutrophils exposed to biofilm bacteria or the BHI media control (Figure 16).

Figure 15. Quantification of neutrophils present on Transwell membrane.

Neutrophils ($5x10^6$cells/ml) were placed in the upper chamber of a Transwell cassette with planktonic bacteria, biofilm bacteria or BHI underneath. Following a 1 hour incubation at 37ºC + 5% CO_2, the quantity of neutrophils on the membrane was assessed based on MPO activity. Fluorescence was measured at excitation 530 nm and emission 590 nm and a standard curve of MPO activity was used to generate values in mU/mL. The data shown are mean MPO activities +/- SEM. There were no significant differences in neutrophil quantity on the membrane. Statistical significance was assessed by the non-parametric KW test. n=15.

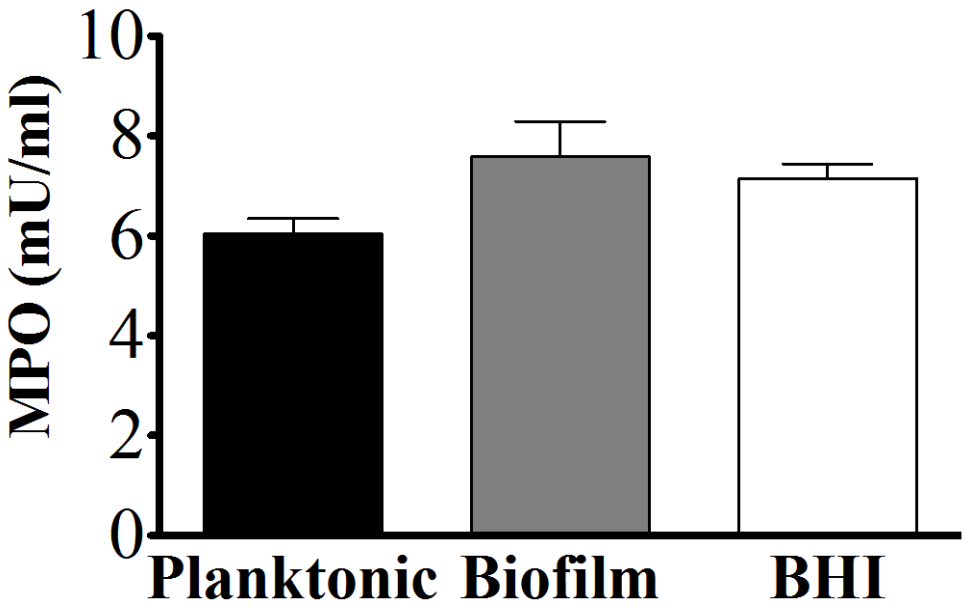
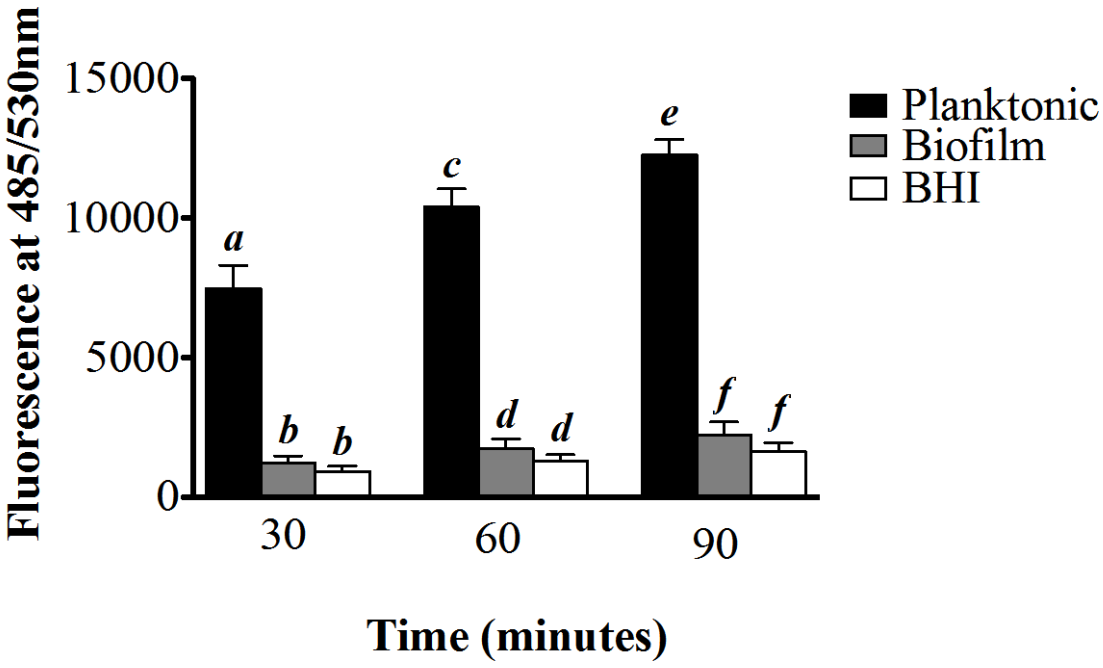

MPO (mU/ml)
10
8
6
4
2
0
Planktonic
Biofilm
BHI

Figure 16. Neutrophil oxidative activity when exposed to planktonic bacteria, biofilm bacteria or the BHI media control across the Transwell membrane.

Neutrophils ($5x10^6$cells/ml) were placed in the upper chamber of a Transwell cassette with planktonic bacteria, biofilm bacteria or BHI underneath. Following a 1 hour incubation at 37^oC + 5% CO_2, the oxidative activity of the neutrophils on the membrane was assessed based on hydrogen peroxide production. Fluorescence was measured at excitation 485 nm and emission 530 nm over a time course of 3 hours, taking measurements every 30 minutes. The data shown are mean fluorescence values +/- SEM at 30, 60 and 90 minutes. At all time points, neutrophils exposed to planktonic bacteria show significantly higher oxidative activity than neutrophils exposed to biofilm bacteria or BHI across the membrane (***a-b, c-d, e-f***). There was no difference between neutrophils exposed to ECFs from biofilm bacteria and the BHI medium control. Statistical significance was assessed by ANOVA. ***a-f*** $p<0.05$, n=9.


Fluorescence at 485/530nm
15000
10000
5000
0
a
b
b
c
d
d
e
f
f
30
60
90
Time (minutes)
Planktonic
Biofilm
BHI

3.4 Neutrophils exhibit different levels of oxidative activity when directly exposed to the growth media containing ECFs from planktonic bacteria, biofilm bacteria or BHI

The previous experiment demonstrated that the neutrophils that were placed in the upper chamber of a Transwell with planktonic bacteria below the membrane exhibited higher oxidative activity than the neutrophils that were placed above biofilm bacteria or BHI media. The membrane separating the neutrophils from these bacterial samples had a pore size of 0.4 μm, which was too small for either *F. necrophorum* or *P. levii* to cross the membrane (this was verified, data not shown). Therefore the next set of experiments focused on the ECFs present in the growth media from planktonic bacteria or biofilm bacteria. The planktonic cultures and biofilms were grown as described and then the media that the bacteria were growing in was centrifuged and filtered to remove live bacteria, leaving only the growth media containing the ECFs. Neutrophils were exposed directly to these ECFs from planktonic bacteria, biofilm bacteria or BHI and assessed for oxidative burst. Oxidative burst was measured kinetically using the DCF assay and as a single endpoint using the NBT assay (Figures 17, 18). As observed in the experiments across the Transwell membranes, the neutrophils that were exposed directly to the growth medium containing ECFs from planktonic bacteria exhibited significantly higher levels of activation than neutrophils exposed directly to ECFs from biofilm bacteria or the BHI medium control (Figures 17, 18). There was no difference in oxidative activity between neutrophils exposed to ECFs from biofilm bacteria and neutrophils exposed to the BHI media control (Figures 17, 18).

Figure 17. Neutrophil oxidative activity after direct exposure to the growth media containing ECFs from planktonic bacteria, biofilm bacteria or the BHI media control

Neutrophils ($6x10^6$cells/mL) were incubated directly with the growth media containing ECFs from planktonic bacteria, biofilm bacteria or BHI. Hydrogen peroxide production was assessed by the DCF assay as a measure of neutrophil oxidative activity. Fluorescence was measured at excitation 485 nm and emission 530 nm over a time course of 2 hours, taking measurements every 20 minutes. The data shown are mean fluorescence values +/- SEM at 20, 60 and 100 minutes. At all time points, neutrophils exposed to the ECFs from planktonic bacteria show significantly higher oxidative activity than neutrophils exposed to the ECFs from biofilm bacteria or BHI (***a-c, d-f, g-i***). Statistical significance was assessed by ANOVA. ***a-i*** $p<0.05$, n=27.

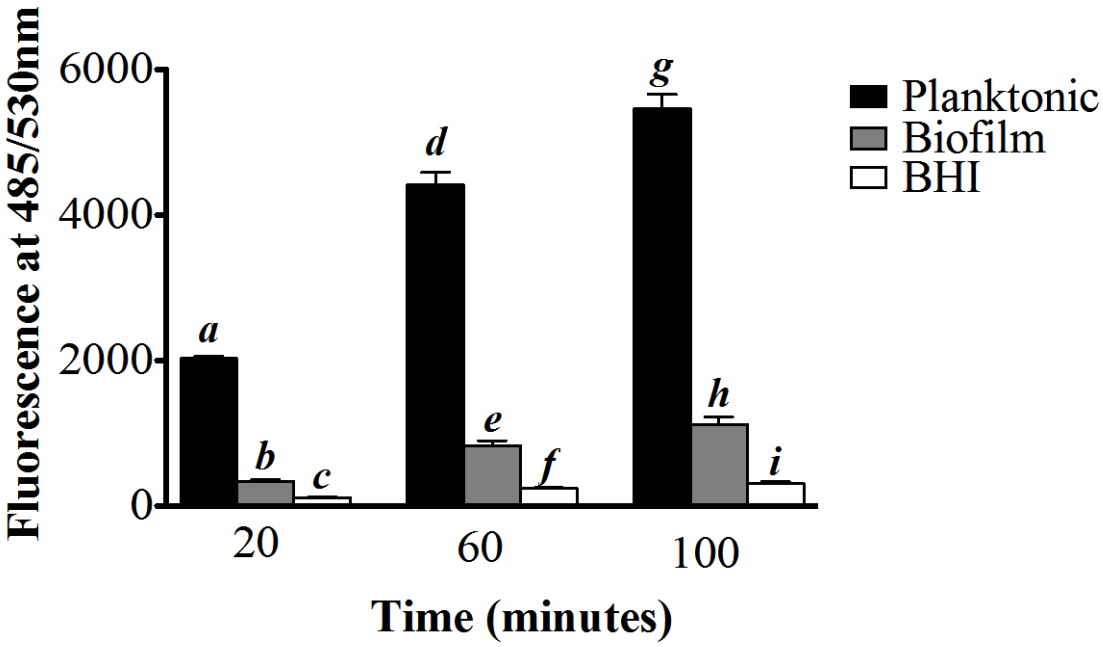
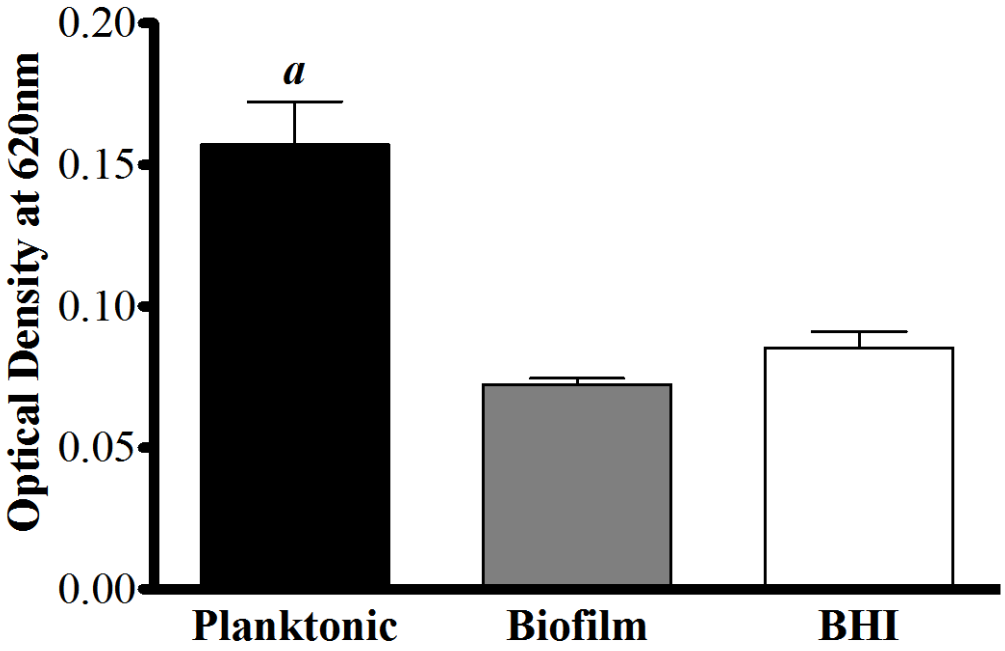

Fluorescence at 485/530nm
6000
4000
2000
0
a
b
c
d
e
f
g
h
i
20
60
100
Time (minutes)
Planktonic
Biofilm
BHI

Figure 18. Neutrophil oxidative activity after direct exposure to the growth media containing ECFs from planktonic bacteria, biofilm bacteria or the BHI medium control

Neutrophils ($7x10^6$cells/mL) were incubated with the growth media containing ECFs from planktonic bacteria, biofilm bacteria or BHI for 1 hour at 37°C + 5% CO_2 in the presence of NBT. Measuring the optical density at 620 nm assessed oxidative burst. Data were expressed as mean absorbance +/- SEM. Neutrophils exposed to the ECFs from planktonic bacteria show significantly higher oxidative activity than neutrophils exposed to the ECFs from biofilm bacteria or BHI (***a***). There was no difference in oxidative activity between neutrophils that were exposed to ECFs from biofilm bacteria and the BHI medium control. Statistical significance was assessed by the non-parametric KW test. ***a*** $p<0.05$, n=12.


Optical Density at 620nm
0.20
0.15
0.10
0.05
0.00
a
Planktonic
Biofilm
BHI

3.5 The growth medium containing ECFs from planktonic bacteria is significantly more cytotoxic than the growth medium containing ECFs from biofilm bacteria or the BHI medium control

The next experiment was performed to investigate the cytotoxic effects the extracellular factors had on neutrophils. Cytotoxicity was assessed by measuring the amount of LDH released into the supernatant after incubating the neutrophils with the extracellular factors for 1 hour at 37ºC +5% CO_2. Incubation with 2.5% Triton X-100 served as a total cell lysis (positive) control and data were expressed as mean absorbance at 492 nm relative to this positive control (Figure 19).

3.6 The growth medium containing ECFs from biofilm bacteria contains significantly more protein and LPS than the growth medium containing ECFs from planktonic bacteria

The next experiments were designed to investigate the composition of the ECFs from planktonic bacteria and biofilm bacteria and determine the quantity of the neutrophil stimulating molecule LPS. The protein concentrations of the growth media from planktonic bacteria and biofilm bacteria were assessed using a BioRad protein assay. The growth medium from biofilm bacteria contained significantly more protein than growth medium from planktonic bacteria (Figure 20). LPS concentrations in the growth media containing ECFs from planktonic bacteria and biofilm bacteria were assessed with the sensitive LAL endotoxin quantitation assay. The results of the LPS quantitation indicated that the growth medium from biofilm bacteria contained significantly more LPS than the growth medium containing ECFs from planktonic bacteria (Figure 21).

Figure 19. Neutrophils released significantly more LDH due to cell lysis when exposed to the growth medium containing ECFs from planktonic bacteria compared to ECFs from biofilm bacteria and the BHI control.

Neutrophils ($7x10^6$cells/mL) were incubated with the growth media containing ECFs from planktonic bacteria, biofilm bacteria or BHI for 1 hour at 37ºC + 5% CO_2. Data represent mean absorbance values at 492 nm relative to treatment with 2.5% Triton X-100 (complete cellular lysis; dotted line) +/- SEM. Neutrophil lysis was significantly higher when exposed to ECFs from planktonic bacteria than when exposed to ECFs from biofilm bacteria and the BHI medium control (***a***). There was no difference in lysis between neutrophils that were exposed to ECFs from biofilm bacteria and the BHI medium control. Statistical significance was assessed by ANOVA. ***a*** $p<0.05$, n=12.

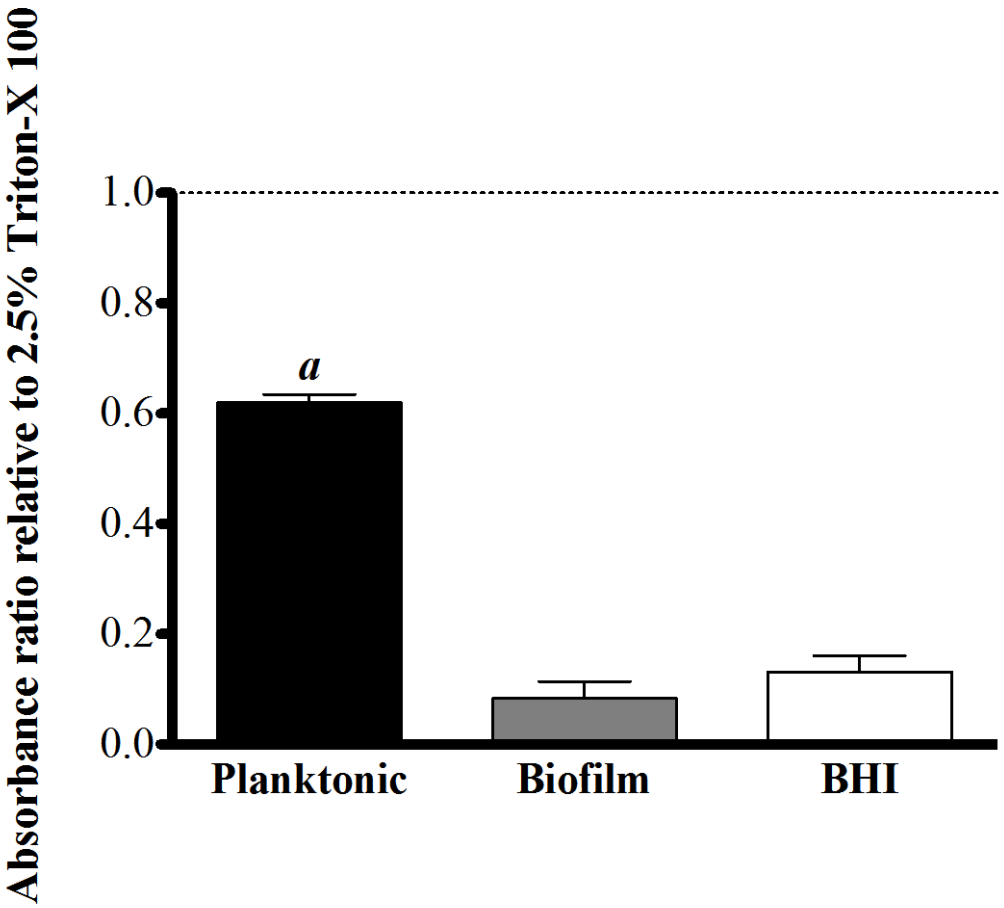

Absorbance ratio relative to 2.5% Triton-X 100
1.0
0.8
0.6
0.4
0.2
0.0
a
Planktonic
Biofilm
BHI

Figure 20. Protein concentrations of filtered growth media containing ECFs from planktonic bacteria and biofilm bacteria.

Planktonic bacteria and biofilm bacteria were prepared, centrifuged at 3200 x *g* at 4ºC and passed through a 0.2 μm filter. Protein concentrations were assessed using a microplate BioRad assay and BSA was used as the protein standard to generate a standard curve. The BHI medium was assessed for protein concentration as well. The data represent mean protein concentrations as determined by the standard curve +/- SEM. The protein concentrations in ECFs from biofilm bacteria were significantly higher than the ECFs from planktonic bacteria and the BHI media control (***a***). There was no difference between the ECFs from planktonic bacteria and the BHI control. Statistical significance was assessed by ANOVA. ***a*** $p<0.05$, n=6

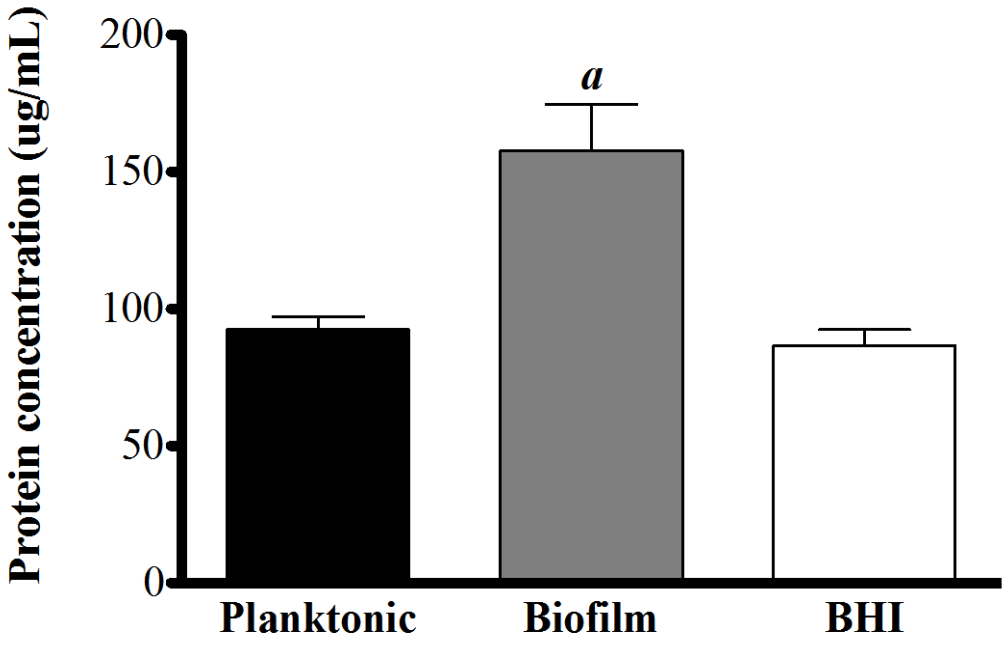
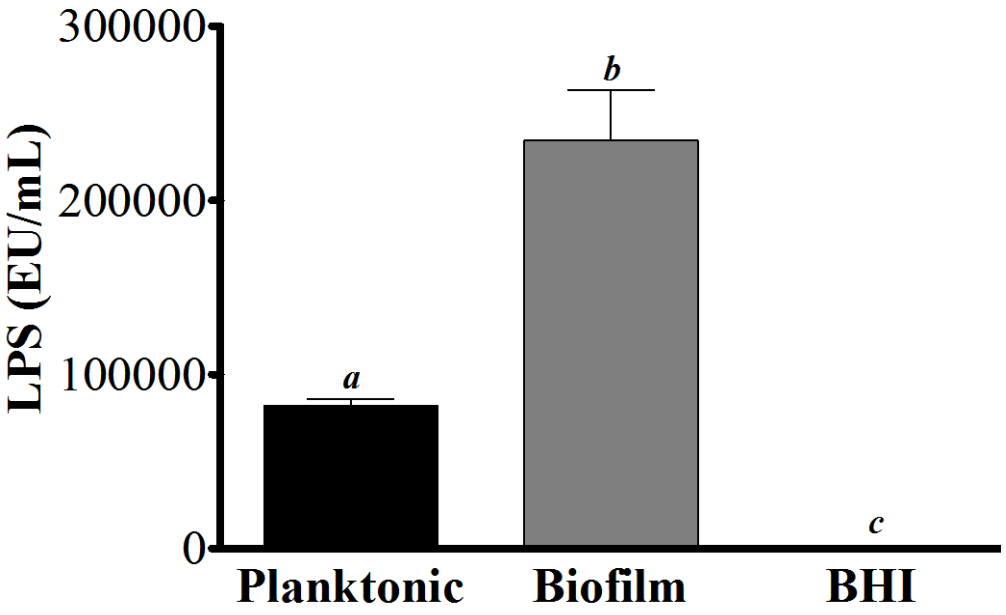

Protein concentration (ug/mL)
200
150
100
50
0
a
Planktonic
Biofilm
BHI

Figure 21. LPS concentrations in filtered growth media containing ECFs from planktonic bacteria and biofilm bacteria.

Planktonic bacteria and biofilm bacteria were prepared, centrifuged at 3200 x *g* at 4ºC and passed through a 0.2 µm filter. LPS concentration was assessed using an LAL endotoxin quantitation kit and an *E. coli* endotoxin standard was used to generate the standard curve. BHI was assessed for LPS as a negative control. The data represent mean LPS concentration in endotoxin units/mL (EU/mL) as determined by the standard curve +/- SEM. ECFs from biofilm bacteria contained significantly more LPS than ECFs from planktonic bacteria and the BHI control (***a-c***). ECFs from planktonic bacteria contained more LPS than the BHI medium control (***a,c***). Statistical significance was assessed by ANOVA. ***a-c*** $p<0.05$, n=8


300000
200000
100000
0
LPS (EU/mL)
a
b
c
Planktonic
Biofilm
BHI

3.7 Removal of LPS from the bacterial growth media results in changes to the cytotoxic effects and differences in oxidative burst when neutrophils are exposed to LPS-reduced samples

The planktonic growth media elicited a significantly stronger oxidative response from the neutrophils and was more cytotoxic to neutrophils than the biofilm growth media (Figures 17, 19). The next experiments investigated the neutrophil oxidative response and the cytotoxicity of the growth media when LPS was removed using an endotoxin removal resin. Following incubation with the removal resin, the fractions were assessed for LPS concentrations with the LAL assay and it was evident that the resin was extremely efficient at removing endotoxin (Figure 22). These LPS-reduced fractions were then incubated directly with the neutrophils and the neutrophils were assessed for oxidative burst and cell death (due to cytotoxicity of the fractions). The results indicate that the removal of LPS from planktonic growth medium significantly lowered the neutrophil oxidative response when compared with unaltered planktonic growth medium (Table 1, Figure 23). Removal of LPS from biofilm growth media significantly increased the neutrophil oxidative response when compared with unaltered biofilm growth media (Table 1, Figure 23). There was no difference in oxidative activity when neutrophils were exposed to LPS-reduced planktonic ECFs compared to neutrophils that were exposed to LPS-reduced biofilm ECFs (Table 1, Figure 23).

The removal of LPS also resulted in changes to the cytotoxicity of the growth media containing ECFs from planktonic bacteria and biofilm bacteria. Removal of LPS from the planktonic growth medium resulted in a significant reduction in cytotoxicity to neutrophils (Figure 24). Removal of LPS from the biofilm growth medium resulted in a

Figure 22. LPS concentrations before and after treatment with LPS-removal resin.

Planktonic bacteria and biofilm bacteria were prepared, centrifuged at 3200 x *g* at 4ºC and passed through a 0.2 μm filter. LPS was removed from the samples by 3 consecutive incubations with endotoxin removal resin. LPS concentration was assessed using an LAL endotoxin quantitation kit and an *E. coli* endotoxin standard was used to generate a standard curve. BHI was assessed for LPS as a negative control. LR = LPS reduced. The data represent mean LPS concentration in endotoxin units/mL (EU/mL) as determined by the standard curve +/- SEM. ECFs from biofilm bacteria contained significantly more LPS than ECFs from planktonic bacteria (***a, c***). ECFs from planktonic bacteria contained more LPS than the BHI medium control as well as both planktonic and biofilm LPS-reduced samples (***a, b***). There were no differences in LPS-reduced samples or the BHI control (***b***). Statistical significance was assessed by ANOVA. ***a-c*** $p<0.05$, n=6-12.

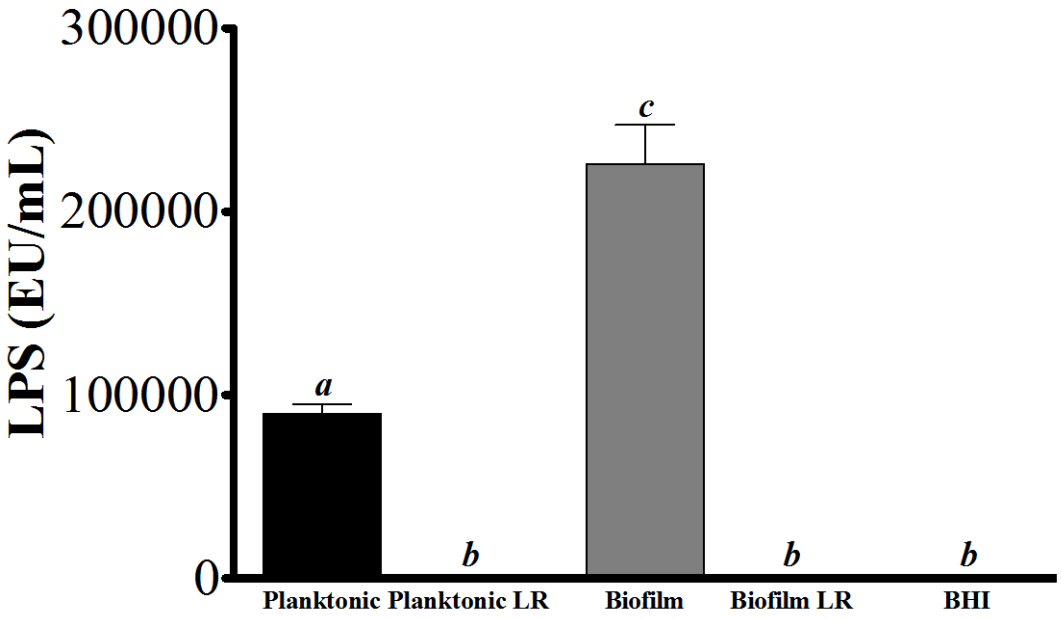

300000
200000
100000
0
LPS (EU/mL)
a
b
c
b
b
Planktonic
Planktonic LR
Biofilm
Biofilm LR
BHI

Table 1. Neutrophil activity after direct exposure to the LPS-reduced ECFs from planktonic bacteria or biofilm bacteria.

Planktonic bacteria and biofilm bacteria were prepared, centrifuged at 3200 x *g* at 4ºC and passed through a 0.2 μm filter. LPS was removed from the samples by 3 consecutive incubations with endotoxin removal resin and then neutrophils were exposed to both unaltered ECFs or LPS-reduced samples. LR = LPS-reduced. The data shown are mean fluorescence values +/- SEM at 20, 60 and 100 minutes. Neutrophils exposed to the ECFs from planktonic bacteria show significantly higher oxidative activity than neutrophils exposed to the ECFs from biofilm bacteria, BHI or LPS-reduced ECFs from planktonic bacteria and biofilm bacteria (***a-d***). At all time points there was no difference in the oxidative activity of neutrophils exposed to LPS-reduced ECFs from either planktonic bacteria or biofilm bacteria, indicated by having the same letter down the columns (***b***). Statistical significance was assessed by ANOVA. ***a-d*** $p<0.05$. n=9.

	Mean Fluorescence at 485/530 nm at Times (min) +/- SEM		
	20	**60**	**100**
Planktonic	2060 +/- 47.7 ***a***	4251 +/- 269.9 ***a***	4893 +/- 325.2 ***a***
Planktonic LR	676.2 +/- 70.4 ***b***	2198 +/- 176.1 ***b***	3042 +/- 227.7 ***b***
Biofilm	366.3 +/- 22.8 ***c***	1146 +/- 111.9 ***c***	1718 +/- 164.4 ***c***
Biofilm LR	815.9 +/- 80.7 ***b***	2340 +/- 205.9 ***b***	3366 +/- 230.7 ***b***
BHI	144.7 +/- 17.3 ***d***	283.5 +/- 41.6 ***d***	383.8 +/- 54 ***d***

Figure 23. Neutrophil activity after direct exposure to the LPS-reduced ECFs from planktonic bacteria or biofilm bacteria.

Planktonic bacteria and biofilm bacteria were prepared, centrifuged at 3200 x *g* at 4^{o}C and passed through a 0.2 μm filter. LPS was removed from the samples by 3 consecutive incubations with endotoxin removal resin and then neutrophils were exposed to both unaltered ECFs or LPS-reduced samples. LR = LPS-reduced. The data shown are mean fluorescence values +/- SEM at 60 minutes. Neutrophils exposed to the ECFs from planktonic bacteria show significantly higher oxidative activity than neutrophils exposed to the ECFs from biofilm bacteria, BHI or LPS-reduced ECFs from planktonic bacteria and biofilm bacteria (***a-d***). There was no difference in the oxidative activity of neutrophils exposed to LPS-reduced ECFs from either planktonic bacteria or biofilm bacteria (***b***). Statistical significance was assessed by ANOVA. ***a-d*** $p<0.05$. n=9.

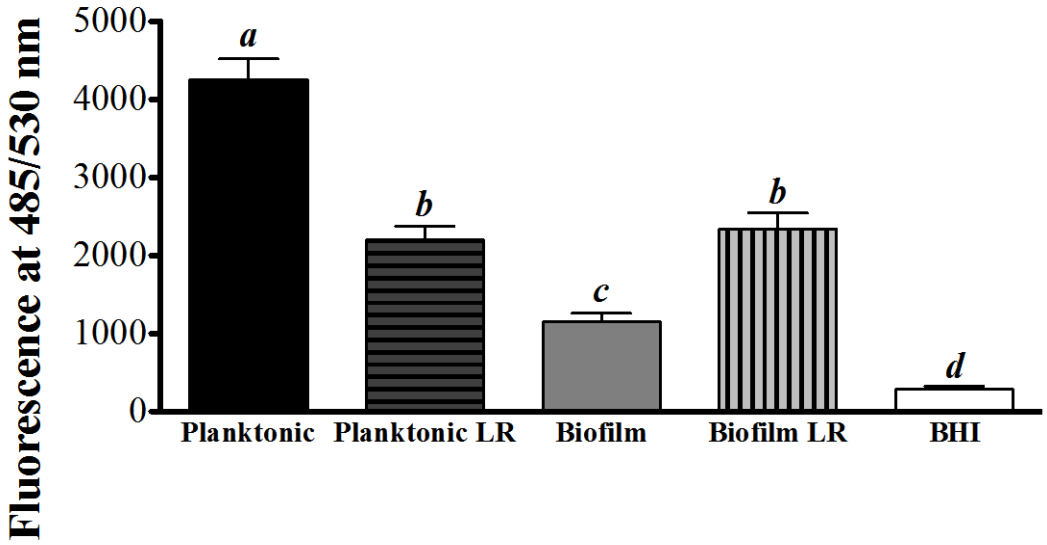
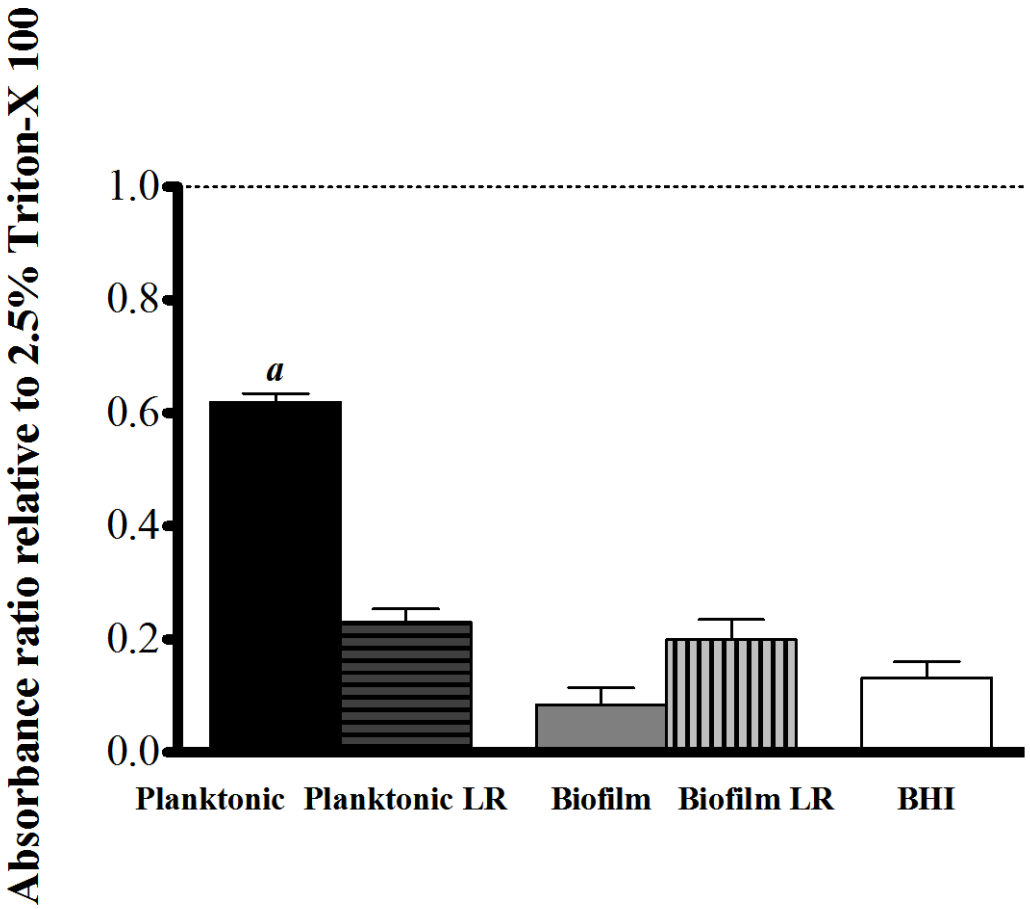

Fluorescence at 485/530 nm
5000
4000
3000
2000
1000
0
a
b
c
b
d
Planktonic
Planktonic LR
Biofilm
Biofilm LR
BHI

Figure 24. Removal of LPS results in a significant reduction in the cytotoxicity of the ECFs from planktonic bacteria.

Neutrophils ($7x10^6$cells/mL) were incubated with unaltered ECFs or LPS-reduced samples. LR = LPS-reduced. Data represent mean absorbance values at 492 nm relative to treatment with 2.5% Triton X-100 (complete cellular lysis; dotted line) +/- SEM. Neutrophil lysis was significantly higher when exposed to ECFs from planktonic bacteria than when exposed to ECFs from biofilm bacteria, LPS-reduced ECFs from either planktonic bacteria or biofilm bacteria or the BHI medium control (***a***). Statistical significance was assessed by ANOVA. ***a*** $p<0.05$, n=6-12


Absorbance ratio relative to 2.5% Triton-X 100
1.0
0.8
0.6
0.4
0.2
0.0
a
Planktonic
Planktonic LR
Biofilm
Biofilm LR
BHI

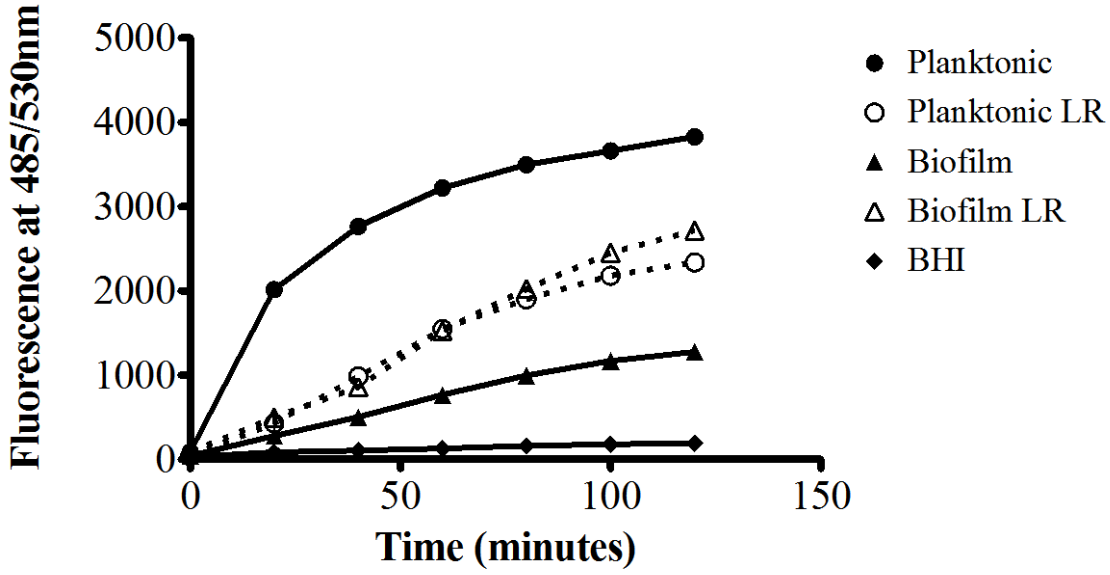
slight increase in cytotoxicity. As observed with oxidative activity in the previous experiment, there was no difference in cytotoxicity when neutrophils were exposed to LPS-reduced planktonic ECFs or LPS-reduced biofilm ECFs (Figure 24).

To further investigate the effect of removing LPS from the extracellular fractions, the samples were treated with PMB to neutralize the LPS. The addition of PMB to the extracellular factors prior to incubation with neutrophils resulted in oxidative responses that were similar to those observed when LPS was removed with the resin (Figures 25A, 25B). The neutrophils that were exposed to PMB-treated planktonic factors showed a significant decrease in oxidative activity compared to the neutrophils that were exposed to unaltered planktonic ECFs (Figure 25B). The neutrophils that were exposed to PMB-treated biofilm ECFs showed an increase in oxidative activity compared to unaltered biofilm ECFs (Figure 25B) As observed with LPS-removal, when LPS was neutralized the neutrophils responded with the same intensity to both planktonic and biofilm ECFs (Figures 23, 25A, 25B).

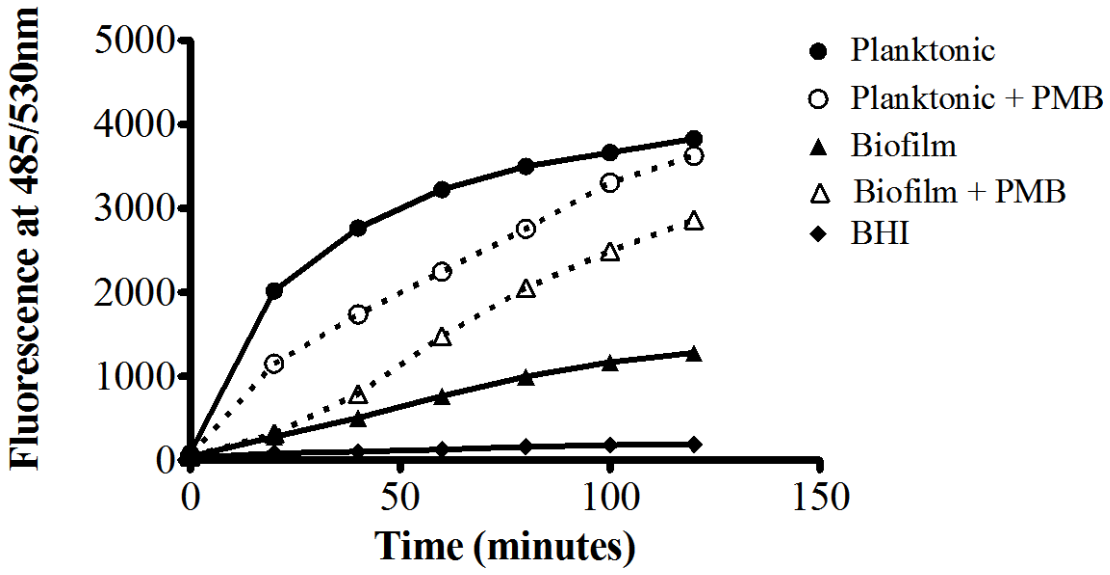
Figure 25. Neutralization of LPS by polymyxin B results in changes to neutrophil oxidative responses

Neutrophils ($6x10^6$cells/mL) were incubated with unaltered extracellular factors, samples treated with LPS-removal resin (23A) or samples treated with polymyxin B (23B). The neutrophils were then assessed for oxidative activity based on hydrogen peroxide production with the DCF assay. The data shown are mean fluorescence values recorded every 20 minutes for 2 hours. LR= LPS-reduced, PMB= polymyxin B. n=3.

A.


Fluorescence at 485/530nm
5000
4000
3000
2000
1000
0
0
50
100
150
Time (minutes)
Planktonic
Planktonic LR
Biofilm
Biofilm LR
BHI

B.


Fluorescence at 485/530nm
5000
4000
3000
2000
1000
0
0
50
100
150
Time (minutes)
Planktonic
Planktonic + PMB
Biofilm
Biofilm + PMB
BHI

DISCUSSION

4.1 Biofilm characterization

The first objective of this project was to grow mixed-species anaerobic biofilms on a substrate (polycarbonate) that to our knowledge has not been used before for the generation of this type of biofilm. Many studies have shown that it is possible to grow-mixed species biofilms on the pegs of a Calgary Biofilm Device (CBD) lid, and an adaptation of the protocol designed by Ceri and colleagues (1999) was used to generate biofilms composed of *F. necrophorum* and *P. levii* on the underside of Transwell membranes (Ceri *et al.,* 1999; Golby *et al.*, 2012). Viable bacterial cell counts demonstrated that the two bacteria remained in approximately a 1:1 ratio after 12 days of growth under anaerobic conditions (Figure 12). The results of confocal microscopy on biofilms stained with the general bacterial stain (AO) demonstrated the uniform thickness of the mixed-species biofilms used in this study (Figure 13A). AO is a membrane permeable nucleic acid stain; therefore it can stain both viable and non-viable bacteria to give an overall depth of the biofilms generated on the membrane (Kepner and Pratt, 1994). On average the thickness of the biofilms was approximately 60 μm and this correlates well with published literature regarding the depth of viable biofilms composed of similar bacteria as the anaerobes in this study (Figure 13B; Hope and Wilson, 2003).

Scanning electron microscopy was carried out on the mixed-species biofilms to verify uniformity on the membrane and that the biofilms were composed of the morphotypes typical of the two bacterial species of interest (Figure 14). A sterile membrane was imaged as a negative control (Figure 14A). The images at 100 and 1000 times magnification showed that the biofilms were uniformly distributed on the

Transwell membrane and the 10,000 times magnification indicated that they were composed of the morphotypes of *F. necrophorum* (long fusiform rods) and *P. levii* (short coccobacilli) (Figure 14B). It is important to note here that the biofilms have been desiccated to prepare them for imaging; this will greatly reduce the thickness of the extracellular polysaccharides and deform the biofilm (Priester *et al.,* 2007; Figure 14B). Therefore the results of confocal are used for determining the depth of the biofilm and SEM simply indicates the morphology of bacteria present in the biofilm. These results confirm our first hypothesis and indicate that it is possible to generate mixed-species anaerobic biofilms on a polycarbonate membrane in the Transwell cassette system. To our knowledge this is the first time that biofilms composed of *F. necrophorum* and *P. levii* have been generated *in vitro*. This method of biofilm growth can be used in a variety of experiments to examine both bacterial characteristics and the response of host immune cells to the biofilms.

4.2 Experiments across the membrane

The initial experiments were carried out using the Transwell cassette system, which evaluated neutrophil responses to any ECFs in the growth medium from planktonic bacteria or biofilm bacteria that were able to cross the 0.4 μm membrane. The MPO assay introduces hydrogen peroxide (H_2O_2) to be broken down by the MPO present in the PMNs and this generates an oxygen radical that combines with a hydrogen donor (AH_2) and creates a coloured compound (A) (Bradley *et al.,* 1982; Quade and Roth, 1997). The amount of coloured compound generated corresponds to the number of neutrophils present in the sample. The whole membrane was removed from each Transwell system in order to have a standardized area for all samples tested. By measuring MPO activity, we

determined that there was no difference in the amount of neutrophils attached to or embedded within the membrane when planktonic bacteria, biofilm bacteria or BHI media were placed in the lower compartment of the Transwell (Figure 15).

The experimental design (without contact between the neutrophils and bacteria) eliminates the argument that the EPS produced by biofilms is acting as a physical barrier and provides evidence that the soluble factors present in the bacterial growth media are participating in neutrophil activation. The activity (oxidative burst) of these neutrophils was then assessed with the DCF assay. It was immediately evident that the neutrophils that were placed on membranes with planktonic cultures underneath generated a much more pronounced oxidative burst than the neutrophils placed above biofilm bacteria or the BHI medium control (Figure 16). Our results demonstrated that ECFs from the planktonic culture were able to cross the Transwell membrane and stimulate the neutrophils to produce reactive oxygen species. Neutrophils that were exposed to biofilm bacterial products did not show this level of activation; in fact they behaved similarly to the neutrophils that were exposed to the control (BHI medium) (Figure 16). These findings are contrary to published literature that indicates neutrophils can be stimulated by the soluble compounds in the supernatants from mature biofilms (Gunther *et al.,* 2007). Research from the early 1990s demonstrated that the oxidative burst of neutrophils exposed to biofilms was 75% less than the oxidative burst of neutrophils exposed to planktonic cells of the same species (*P. aeruginosa*) and the results shown here correlate with those findings (Jensen *et al.,* 1990; Jensen *et al.,* 1992; Figure 16). One potential explanation for the observed result is that the biofilm extracellular polysaccharides can act as a trap and prevent the soluble compounds from diffusing through the membrane,

essentially acting as a diffusion barrier similar to what has been observed with antimicrobial diffusion into the biofilm (Costerton *et al.,* 1999; Gunther *et al.*, 2009). Another possibility for the lack of oxidative burst when exposed to biofilms is that the biofilms do not produce as many neutrophil-stimulating molecules as the planktonic cultures, however the literature suggests that this is unlikely (Yeom *et al.,* 2012). Biofilms have been shown to release soluble bacterial products into the surrounding environment, causing chronic inflammation therefore it is expected that the anaerobic biofilms used in this study would release various molecules into the growth medium as well (Oscarsson *et al.,* 2008). The published literature combined with our experimental results lead to the development of the next set of experiments.

4.3 Direct exposure of neutrophils to the extracellular compounds in planktonic and biofilm growth media

Experiments where the neutrophils were exposed to planktonic bacteria and biofilm bacteria across the Transwell membrane indicated that one or more compounds in the growth media were capable of crossing the membrane to stimulate neutrophils. The next line of investigation involved the direct exposure of neutrophils to the growth media containing ECFs after the removal of all viable bacteria. The results of direct exposure correlated with the data from experiments across the membrane, where the neutrophils that were exposed to the growth medium containing ECFs from planktonic growth demonstrated a much greater oxidative burst than the neutrophils that were exposed to growth medium from biofilm bacteria or the BHI medium control (Figure 17). This result was contrary to the hypothesis that neutrophils exposed growth media containing ECFs from biofilm bacteria would exhibit a more oxidative burst than those exposed to ECFs

from planktonic bacteria. The result was confirmed using the NBT assay as a secondary, endpoint experiment (Figure 18). This limited response to biofilm bacteria could explain the difficulties neutrophils encounter with biofilm clearance *in vivo* (Gunther *et al.,* 2007). The oxidative response of the neutrophils that were exposed to the growth medium containing ECFs from planktonic bacteria can be explained by the presence of numerous bacterial products such as LPS, leukotoxin and SCFA, however these molecules should also be present in the biofilm supernatant, therefore the lack of activity in neutrophils exposed to biofilms was unexpected (Carretta *et al.,* 2012; Mills *et al.,* 2006; Nagaraja *et al.,* 2005). According to published literature, there can be dramatic differences in the amount of cytokines produced by monocytes exposed to planktonic bacteria compared to biofilm bacteria of the same species (Ciornei *et al.,* 2010). This depends on the species of bacteria; for example it was shown that planktonic bacteria and biofilm bacteria of *S. aureus* induced similar cytokine production, whereas biofilms of *P. aeruginosa* induced greater cytokine production than planktonic *P. aeruginosa* (Ciornei *et al.,* 2010). Species-specific differences should not be an issue in the current experiments, as both planktonic and biofilm samples were prepared using a 1:1 ratio of the same bacteria, therefore the differences in neutrophil activation observed can be attributed to the mode of growth (planktonic compared to biofilm).

The cytotoxic effect of these cell-free supernatants was also investigated by measuring LDH release from lysed neutrophils and the planktonic ECFs were shown to be much more cytotoxic than the biofilm ECFs (Figure 19). The presence of *F. necrophorum* leukotoxin and various other compounds can explain the cytotoxicity of the

growth medium containing ECFs from planktonic bacteria, but we expect these to be present in the growth medium containing ECFs from biofilm bacteria as well.

The *F. necrophorum* leukotoxin (LKT) appears to have specific activity towards bovine PMNs, as studies have shown that the toxin does not affect swine or rabbit neutrophils (Narayanan *et al.,* 2002). Even at low concentrations, LKT can cause neutrophil activation and induce apoptosis, but at higher concentrations it can cause immediate cell death through necrosis (Narayanan *et al.,* 2002). Induction of apoptosis or necrosis is an extremely important mechanism for pathogenesis, as this will help the bacteria avoid destruction by the host neutrophils. For these reasons, the concentration of LKT in the growth media containing ECFs from planktonic bacteria and biofilm bacteria will need to be investigated in the future. It has been documented that gene expression in planktonic bacteria can differ dramatically from biofilm bacteria of the same species therefore we expect there will be differences in the overall production of LKT from one mode of growth to the other (Fux *et al.,* 2005). This phenomenon could help explain the difficulties of biofilm-clearance observed in many infections.

The ECFs from planktonic bacteria and biofilm bacteria were investigated to look for potential sources for the differences observed in cytotoxicity and neutrophil oxidative activity. Cultures of planktonic and biofilm bacteria consisting of similar viable numbers were processed and the protein concentration of each was determined. The results demonstrated that the growth medium with ECFs from biofilm bacteria contained more protein than the growth medium with ECFs from planktonic bacteria (Figure 20). Given the length of time it takes to grow biofilms and the higher levels of expression of various genes observed in biofilm growth compared to planktonic bacteria this is not surprising.

As mentioned in Section 1.5, increased expression of certain proteins is observed in biofilm growth compared to their planktonic counterparts (ex. outer membrane porins like OmpC) (White, 2006; Yeom *et al.,* 2012; Fux *et al.,* 2005). This is not restricted to membrane proteins or the increased production of exopolysccharides, many other genes are up regulated, resulting in phenotypes that differ dramatically from bacteria grown as planktonic cultures (Stoodley *et al.,* 2002). For these reasons the increase in protein concentration observed in ECFs from biofilm bacteria compared to planktonic bacteria was anticipated (Figure 20). More protein in the biofilm samples does correlate with published literature, however when neutrophils were exposed to the ECFs from planktonic bacteria and biofilm bacteria it was expected that the biofilm-exposed neutrophils would show higher levels of activation due to the higher protein concentration and this was not the case (Figures 17, 18). The soluble proteins from various bacterial species such as *Helicobacter pylori* have been shown to be potent stimuli for neutrophils; therefore the lack of neutrophil response to the growth medium containing ECFs from biofilm bacteria was unexpected (Shimoyama *et al.,* 2003; Kim *et al.,* 1998). Since the differences in protein concentration could not account for the differences in oxidative burst observed when neutrophils were exposed to growth media containing ECFs from planktonic bacteria compared to biofilm bacteria, the next experiments investigated the amount of LPS in each sample and the effect this had on the neutrophils.

4.4 LPS and the effect of LPS-reduction on neutrophil oxidative burst

The concentration of LPS in the growth media containing ECFs from planktonic bacterial and biofilm bacteria was determined using an LAL endotoxin quantitation assay.

Based on the results of the oxidative burst assay, where neutrophils were stimulated by planktonic ECFs and not biofilm ECFs, it was expected that the planktonic sample would contain more LPS (a known neutrophil stimulant), however the biofilm sample had significantly more LPS (Figures 17, 21; Doerfler *et al.,* 1989). The amount of LPS present in the biofilm samples could be explained by the up regulation of LPS synthesis genes as discussed in Section 1.5, however the limited neutrophil response to this high concentration of LPS was not anticipated. The alteration of LPS structure by biofilm bacteria is a potential explanation for this observation, however more work will need to be performed on this aspect in the future. In order to investigate this further, experiments where the LPS was removed from both planktonic and biofilm samples were performed. Using an endotoxin removal resin, the LPS that was in each sample was reduced by more than 99%, as advertised by the manufacturer (Figure 22; Pierce Removal resin protocol). Using the same experimental design, neutrophils were exposed to these LPS-reduced ECFs from planktonic bacteria and biofilm bacteria and this changed the neutrophil oxidative bursts (Table 1, Figure 23). There was no significant difference in the responses of neutrophils exposed to LPS-reduced planktonic ECFs compared to neutrophils exposed to LPS-reduced biofilm ECFs and this indicates that LPS plays a significant role in the activation of neutrophils (Table 1, Figure 23). The effect that LPS has on neutrophil activity has been known for many years and is one of the most common stimulatory molecules used when studying neutrophil proinflammatory responses (Ley *et al.,* 2007). Interestingly, when LPS was removed from planktonic samples, the neutrophil response was significantly reduced compared to neutrophils exposed to the unaltered planktonic samples (Table 1, Figure 23). However when LPS was removed from biofilm

samples, the neutrophil response was greater compared to neutrophils exposed to the unaltered biofilm samples (Table 1, Figure 23). It was expected that the removal of LPS from either sample would result in a reduced neutrophil response, as observed in the planktonic samples. The result of LPS-removal from the biofilm samples demonstrated precisely the opposite effect, which suggests that in our experimental conditions another molecule or process participates in overall neutrophil activity, in addition to LPS.

Neutrophil oxidative responses were altered by the removal of LPS from planktonic and biofilm samples, and our next experiment investigated the differences in the cytotoxicity of these LPS-reduced samples. More neutrophils were viable after incubation with LPS-reduced planktonic cell-free supernatants compared with unaltered planktonic samples (Figure 24). This result was interesting and does not correlate with previous work because some literature suggests that neutrophil apoptosis and necrosis can be delayed by the presence of LPS (Sabroe *et al.,* 2003; Yamamoto *et al.,* 1993; Lee *et al.,* 1993). LPS acting as a survival signal is intuitive (if there are still LPS fragments in the environment the neutrophils need to survive to clear all the bacteria producing the LPS), however the opposite was observed when LPS was removed from the growth medium containing ECFs from planktonic bacteria (Figure 24). The LPS-reduced planktonic sample was much less cytotoxic than the unaltered sample, indicating that LPS participates in the cytotoxicity of soluble factors present in the growth media from *F. necrophorum* and *P.levii*. The cytotoxicity results of the biofilm and LPS-reduced biofilm samples were very different than the planktonic results (Figure 24). Despite having massive concentrations of LPS, unaltered biofilm samples caused very little neutrophil lysis (Figures 22, 24). After the LPS was removed, the biofilm samples became slightly

more cytotoxic (Figure 24). LPS acting as a survival signal could be an explanation for the biofilm results (LPS reduction causes more cell death), however this does not account for the trend observed in the planktonic samples (Figure 24). Some literature suggests that monocytes are necessary for LPS to act as a neutrophil survival signal and since these experiments used exclusively purified neutrophils more work will need to be done on this aspect in the future (Sabroe *et al.,* 2003). The results from the planktonic samples indicate that LPS plays an important role in neutrophil activation and lysis, however there must be other factors or toxins playing a role. Some potential examples of these include bacterial DNA, causing neutrophil stimulation due to signaling by TLR9 or the direct action of *F. necrophorum's* leukotoxin itself (Alexopoulou *et al.*, 2001; Nagaraja *et al.,* 2005). As the literature suggests, several TLRs and various other receptors must be simultaneously activated to incite a complete proinflammatory response (Trincheiri *et al.,* 2007)

To ensure that these results were indeed due to the removal of LPS from the samples, separate experiments were conducted using polymyxin B (PMB). PMB was used to neutralize the LPS *in situ* in the growth media containing ECFs from planktonic or biofilm samples prior to exposure to neutrophils. PMB binds to the lipid A component of LPS and by neutralizing the stimulatory moiety, neutrophil responses were altered (Figure 25B). Neutralization of LPS by PMB produced very similar results to those observed in removal of LPS by the resin, where addition of PMB reduced neutrophil oxidative responses to planktonic ECFs and increased neutrophil oxidative responses to biofilm ECFs (Figure 25). The result of this experiment suggests that LPS and lipid A play a role in the activation of neutrophils but since the removal of LPS did not

completely attenuate the neutrophil respiratory burst, there must be other important factors present in the growth media.

4.5 Conclusions

1. Mixed-species anaerobic biofilms of *F, necrophorum* and *P. levii* can be generated on Transwell polycarbonate membranes. This model can be used to study immune responses to biofilm bacteria without the EPS acting as a physical barrier to the cells of interest.
2. Neutrophils that are indirectly exposed to mixed-species planktonic bacteria exhibit a more extensive oxidative response than neutrophils that are exposed to biofilms composed of the same bacteria.
3. ECFs released into the growth medium from planktonic bacteria are important stimulators of neutrophils. The limited neutrophil response to ECFs from biofilm bacteria may explain the reduced ability of the innate immune system to eradicate biofilm-associated infections.
4. Growth medium containing ECFs from mixed-species biofilm bacteria contains significantly more LPS than growth medium containing ECFs from mixed-species planktonic bacteria.
5. LPS plays a major role in the activation of neutrophils, specifically through the interaction of lipid A with TLR4 on the surface of neutrophils. However, it is not the only stimulatory molecule present in the growth media containing ECFs from both mixed-species planktonic bacteria and mixed-species biofilm bacteria that participates in neutrophil activation.

4.6 Future directions

The results of this study indicate that there are significant differences in oxidative responses when neutrophils are exposed to planktonic bacteria compared to neutrophils that are exposed to biofilms composed of the same two species of bacteria. It would be of interest to study the neutrophil responses to monocultures of the planktonic bacteria and biofilms composed of each species individually. This would help determine if either of the two species benefit from growing in co-culture and whether virulence gene expression changes in mixed-species cultures. In the future it would be interesting to assess other parameters of neutrophil activation, such as the differences in transcription of NF-κB dependent genes (eg. IL-1β, IL-6, IL-8). It would also be beneficial to investigate the number of neutrophils that are induced to undergo apoptosis in these types of assays. Chronic inflammation is often observed in biofilm-associated infections; therefore understanding the mechanism behind the neutrophil functional response is crucial. The observations about neutrophil responses to planktonic bacteria compared with biofilm bacteria in this study provide insight into how the biofilm mode of growth presents challenges to the host immune system. This may lead to the development of new treatments for biofilm-associated inflammation.

Further characterization of the growth media containing ECFs from mixed-species anaerobic planktonic bacteria and biofilm bacteria is a logical future direction. One quick experiment to perform would be to heat the ECFs to denature any proteins and then measure neutrophil responses to see if a protein is playing a role in the activation of neutrophils. An alternative experiment that could be performed is to treat the ECFs with a proteinase to degrade them and measure neutrophil responses. As mentioned above, one

molecule of interest is the leukotoxin produced by *F. necrophorum*. It would be ideal to isolate this potent toxin and investigate its effects on neutrophils *in vitro* using the techniques described here. Another interesting line of investigation in the future could be to determine the amount of exogenous bacterial DNA present in the growth media and the potential role it is playing via interaction with TLR9 on the surface of neutrophils.

As mentioned in Section 1.4, some classes of antibiotics have been shown to have immunomodulatory effects and may be able to limit some of the chronic inflammation that is often observed in biofilm-related infections. Studies have shown that macrolide antibiotics may be effective at reducing neutrophil oxidative burst therefore it would be of interest to pre-treat neutrophils with various antibiotics and measure their oxidative responses to bacterial cell-free supernatants using the techniques described here. Some preliminary work has been performed, however no significant differences in neutrophils oxidative burst have been detected in cells treated with antibiotics compared with untreated cells.

Overall the experiments designed in this project provide an interesting model to study neutrophil responses to biofilms. Future studies should focus on further characterization of the ECFs present in the growth media from planktonic bacteria and biofilm bacteria, the mechanism of neutrophil functional responses and the effect that antibiotics have on neutrophil oxidative burst.

REFERENCES

1. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nature Reviews Immunology* **4:** 499-511.

2. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. *Nature* **413:** 732-738.

3. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 9237-9242.

4. Borzecka, K., A. Plociennikowska, H. Bjorkelund, A. Sobota, and K. Kwiatkowska. 2013. CD14 Mediates Binding of High Doses of LPS but Is Dispensable for TNF-alpha Production. *Mediators of Inflammation* **2013:** 12 pages.

5. Brach, M. A., S. Devos, H. J. Gruss, and F. Herrmann. 1992. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell-death. *Blood* **80:** 2920-2924.

6. Bradley, P. P., D. A. Priebat, R. D. Christensen, and G. Rothstein. 1982. Measurement of cutaneous inflammation - estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology* **78:** 206-209.

7. Brook, I. 2002. Microbiology of polymicrobial abscesses and implications for therapy. *Journal of Antimicrobial Chemotherapy* **50:** 805-810.

8. Brunner, J., N. Scheres, N. B. El Idrissi, D. M. Deng, M. L. Laine, A. J. van Winkelhoff, and W. Crielaard. 2010. The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts. *BMC Microbiol* **10:** 5.

9. Buret, A. G. 2010. Immuno-modulation and anti-inflammatory benefits of antibiotics: the example of tilmicosin. *Can J Vet Res* **74:** 1-10.

10. Buret, A., K. H. Ward, M. E. Olson, and J. W. Costerton. 1991. An *in vivo* model to study the pathobiology of infectious biofilms on biomaterial surfaces. *Journal of Biomedical Materials Research* **25:** 865-874.

11. Busch, D. F. 1984. Anaerobes in infections of the head and neck and ear, nose, and throat. *Reviews of Infectious Diseases* **6:** S115-S122.

12. Bylund, J., L. A. Burgess, P. Cescutti, R. K. Ernst, and D. P. Speert. 2006. Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. *Journal of Biological Chemistry* **281:** 2526-2532.

13. Byrne, D. P., J. Potempa, T. Olczak, and J. W. Smalley. 2013. Evidence of mutualism between two periodontal pathogens: co-operative haem acquisition by the HmuY haemophore of *Porphyromonas gingivalis* and the cysteine protease interpain A (InpA) of *Prevotella intermedia*. *Molecular Oral Microbiology* **28:** 219-229.

14. Campanac, C., L. Pineau, A. Payard, G. Baziard-Mouysset, and C. Roques. 2002. Interactions between biocide cationic agents and bacterial biofilms. *Antimicrobial Agents and Chemotherapy* **46:** 1469-1474.

15. Caroff, M., D. Karibian, J. M. Cavaillon, and N. Haeffner-Cavaillon. 2002. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes and Infection* **4:** 915-926.

16. Carretta, M. D., I. Conejeros, M. A. Hidalgo, and R. A. Burgos. 2013. Propionate induces the release of granules from bovine neutrophils. *Journal of Dairy Science* **96:** 2507-2520.

17. Cekici, A., A. Kantarci, H. Hasturk, and T. E. Van Dyke. 2014. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000* **64:** 57-80.

18. Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret. 1999. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology* **37:** 1771-1776.

19. Chatterjee, S. N., and K. Chaudhuri. 2006. Lipopolysaccharides of *Vibrio cholerae*: III. Biological functions. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1762:** 1-16.

20. Chen, W., and H. K. Kuramitsu. 1999. Molecular mechanism for the spontaneous generation of pigmentless *Porphyromonas gingivalis* mutants. *Infection and Immunity* **67:** 4926-4930.

21. Chin, A. C., W. D. Lee, K. A. Murrin, D. W. Morck, J. K. Merrill, P. Dick, and A. G. Buret. 2000. Tilmicosin induces apoptosis in bovine peripheral neutrophils in the presence or in the absence of *Pasteurella haemolytica* and promotes neutrophil phagocytosis by macrophages. *Antimicrobial Agents Chemotherapy* **44:** 2465-2470.

22. Choi, H. S., J. W. Kim, Y.-N. Cha, and C. Kim. 2006. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of Immunoassay & Immunochemistry* **27:** 31-44.

23. Ciornei, C. D., A. Novikov, C. Beloin, C. Fitting, M. Caroff, J. M. Ghigo, J. M. Cavaillon, and M. Adib-Conquy. 2010. Biofilm-forming *Pseudomonas aeruginosa* bacteria undergo lipopolysaccharide structural modifications and induce enhanced inflammatory cytokine response in human monocytes. *Innate Immunity* **16:** 288-301.

24. Clark, R. A. 1999. Activation of the neutrophil respiratory burst oxidase. *Journal of Infectious Diseases* **179:** S309-S317.

25. Colotta, F., F. Re, N. Polentarutti, S. Sozzani, and A. Mantovani. 1992. Modulation of granulocyte survival and programmed cell-death by cytokines and bacterial products. *Blood* **80:** 2012-2020.

26. Cooperstock, M. S. 1974. Inactivation of endotoxin by polymyxin B. *Antimicrobial Agents and Chemotherapy* **6:** 422-425.

27. Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. *Science* **284:** 1318-1322.

28. Danek, J., and U. Zurek. 2014. Changes in domestic animals after endotoxin administration - a review. *Annals of Animal Science* **14:** 479-489.

29. Darveau, R. P. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nature Review Microbiology* **8:** 481-490.

30. Dashper, S. G., K. J. Cross, N. Slakeski, P. Lissel, P. Aulakh, C. Moore, and E. C. Reynolds. 2004. Hemoglobin hydrolysis and heme acquisition by *Porphyromonas gingivalis*. *Oral Microbiology and Immunology* **19:** 50-56.

31. Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery* **2:** 114-122.

32. Decker, T., and M. L. Lohmannmatthes. 1988. A quick and simple method for the quantitation of lactate-dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods* **115:** 61-69.

33. DeLeo, F. R., L. A. Allen, M. Apicella, and W. M. Nauseef. 1999. NADPH oxidase activation and assembly during phagocytosis. *J Immunology* **163:** 6732-6740.

34. Diaz, P. I., P. S. Zilm, and A. H. Rogers. 2002. Fusobacterium nucleatum supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. *Microbiology-Sgm* **148:** 467-472.

35. Dibdin, G. H., S. J. Assinder, W. W. Nichols, and P. A. Lambert. 1996. Mathematical model of beta-lactam penetration into a biofilm of *Pseudomonas aeruginosa* while undergoing simultaneous inactivation by released beta-lactamases. *Journal of Antimicrobial Chemotherapy* **38:** 757-769.

36. Doerfler, M. E., R. L. Danner, J. H. Shelhamer, and J. E. Parrillo. 1989. Bacterial lipopolysaccharides prime human-neutrophils for enhanced production of leukotreien-B4. *Journal of Clinical Investigation* **83:** 970-977.

37. Donelli, G., C. Vuotto, R. Cardines, and P. Mastrantonio. 2012. Biofilm-growing intestinal anaerobic bacteria. *FEMS Immunology and Medical Microbiology* 65: 318-325.

38. Dower, K., D. K. Ellis, K. Saraf, S. A. Jelinsky, and L.-L. Lin. 2008. Innate immune responses to TREM-1 activation: Overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. *Journal of Immunology* **180:** 3520-3534.

39. Durr, M. C., S. A. Kristian, M. Otto, G. Matteoli, P. S. Margolis, J. Trias, K. P. van Kessel, J. A. van Strijp, E. Bohn, R. Landmann, and A. Peschel. 2006. Neutrophil chemotaxis by pathogen-associated molecular patterns - formylated peptides are crucial but not the sole neutrophil attractants produced by *Staphylococcus aureus*. *Cellular Microbiology* **8:** 207-217.

40. Emery, D. L., J. A. Vaughan, B. L. Clark, J. H. Dufty, and D. J. Stewart. 1985. Cultural-characteristics and virulence of strains of *Fusobacterium necrophorum* isolated from the feet of cattle and sheep. *Australian Veterinary Journal* **62:** 43-46.

41. Fischer, C. D., J. K. Beatty, C. G. Zvaigzne, D. W. Morck, M. J. Lucas, and A. G. Buret. 2011. Anti-Inflammatory benefits of antibiotic-induced neutrophil apoptosis: tulathromycin induces caspase-3-dependent neutrophil programmed cell death and inhibits NF-kappaB signaling and CXCL8 transcription. *Antimicrobial Agents Chemotherapy* **55:** 338-348.

42. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappa B involves the toll adapters TRAM and TRIF. *Journal of Experimental Medicine* **198:** 1043-1055.

43. Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley. 2005. Survival strategies of infectious biofilms. *Trends in Microbiology* **13:** 34-40.

44. Golby, S., H. Ceri, L. M. Gieg, I. Chatterjee, L. L. R. Marques, and R. J. Turner. 2012. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. *FEMS Microbiology Ecology* **79:** 240-250.

45. Goldstein, E. J., D. M. Citron, and S. M. Finegold. 1984. Role of anaerobic bacteria in bite-wound infections. *Reviews of infectious diseases* **6** Suppl 1: S177-183.

46. Gunther, F., G. H. Wabnitz, P. Stroh, B. Prior, U. Obst, Y. Samstag, C. Wagner, and G. M. Hansch. 2009. Host defence against *Staphylococcus aureus* biofilms infection: Phagocytosis of biofilms by polymorphonuclear neutrophils (PMN). *Molecular Immunology* **46:** 1805-1813.

47. Hachiya, O., Y. Takeda, H. Miyata, H. Watanabe, T. Yamashita, and F. Sendo. 1995. Inhibition by bacterial lipopolysaccharide of spontaneous and TNF-alpha-induced apoptosis *in vitro*. *Microbiology and Immunology* **39:** 715-723.

48. Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood* **92:** 3007-3017.

49. Hannan, T. J., I. U. Mysorekar, C. S. Hung, M. L. Isaacson-Schmid, and S. J. Hultgren. 2010. Early severe inflammatory responses to uropathogenic *Escherichia coli* predispose to chronic and recurrent urinary tract infection. *PLoS Pathog* **6:** e1001042.

50. Hassett, D. J., T. R. Korfhagen, R. T. Irvin, M. J. Schurr, K. Sauer, G. W. Lau, M. D. Sutton, H. W. Yu, and N. Hoiby. 2010. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. *Expert Opinion on Therapeutic Targets* **14:** 117-130.

51. Herter, J., and A. Zarbock. 2013. Integrin Regulation during Leukocyte Recruitment. *Journal of Immunology* **190:** 4451-4457.

52. Hoiby, N., T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu. 2010. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* **35:** 322-332.

53. Hope, C. K., and M. Wilson. 2003. Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping. *Journal of Microbiological Methods* **54:** 403-410.

54. Jensen, E. T., A. Kharazmi, P. Garred, G. Kronborg, A. Fomsgaard, T. E. Mollnes, and N. Hoiby. 1993. Complement activation by *Pseudomonas aeruginosa* biofilms. *Microbial Pathogenesis* **15:** 377-388.

55. Jensen, E. T., A. Kharazmi, N. Hoiby, and J. W. Costerton. 1992. Some bacterial parameters influencing the neutrophil oxidative burst response to *Pseudomonas aeruginosa* biofilms. *APMIS* **100:** 727-733.

56. Jensen, E. T., A. Kharazmi, K. Lam, J. W. Costerton, and N. Hoiby. 1990. Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. *Infection and Immunity* **58:** 2383-2385.

57. Jousimies-Somer, Summanen P., Citron, D., Baron, E., Wexler, H., Finegold, S. 2002. P.1, 211. Wadsworth Anaerobic Bacteriology Manual. 6th edition. Star Publishing Company, Belmont.

58. Karlsson, A., and C. Dahlgren. 2002. Assembly and activation of the neutrophil NADPH oxidase in granule membranes. *Antioxidants & Redox Signaling* **4:** 49-60.

59. Kepner, R. L., and J. R. Pratt. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples past and present. *Microbiological Reviews* **58:** 603-615.

60. Kim, J. S., H. C. Jung, J. M. Kim, I. S. Song, and C. Y. Kim. 1998. Interleukin-8 expression by human neutrophils activated by *Helicobacter pylori* soluble proteins. *Scandinavian Journal of Gastroenterology* **33:** 1249-1255.

61. Kindt T. J, Goldby R. A, and B. A. Osborne. Innate Immunity. P. 69-71. Kuby Immunology. 6th Edition. WH Freeman, New York.

62. Klein, B. A., E. L. Tenorio, D. W. Lazinski, A. Camilli, M. J. Duncan, and L. T. Hu. 2012. Identification of essential genes of the periodontal pathogen *Porphyromonas gingivalis. BMC Genomics* **13:** 578-595.

63. Korzeniewski, C., and D. M. Callewaert. 1983. An enzyme release assay for natural cytotoxicity. *Journal of Immunological Methods* **64:** 313-320.

64. Krejsa, C. M., and G. L. Schieven. 2000. Detection of oxidative stress in lymphocytes using dichlorodihydrofluorescein diacetate. *Methods in molecular biology* **99:** 35-47.

65. Kumar, A., D. Anderson, R. G. Amachawadi, T. G. Nagaraja, and S. K. Narayanan. 2013. Characterization of *Fusobacterium necrophorum* isolated from llama and alpaca. *Journal of Veterinary Diagnostic Investigation* **25:** 502-507.

66. Kuwano, Y., O. Spelten, H. Zhang, K. Ley, and A. Zarbock. 2010. Rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils. *Blood* **116:** 617-624.

67. Lee, A., M. K. B. Whyte, and C. Haslett. 1993. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *Journal of Leukocyte Biology* **54:** 283-288.

68. Lee, W. D., A. N. Flynn, J. M. LeBlanc, J. K. Merrill, P. Dick, D. W. Morck, and A. G. Buret. 2004. Tilmicosin-induced bovine neutrophil apoptosis is cell-specific and downregulates spontaneous LTB4 synthesis without increasing Fas expression. *Vet Res* **35:** 213-224.

69. Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy* **45:** 999-1007.

70. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology* **7:** 678-689.

71. Lobb, D. A., H. J. Loeman, D. G. Sparrow, and D. W. Morck. 1999. Bovine polymorphonuclear neutrophil-mediated phagocytosis and an immunoglobulin G(2) protease produced by *Porphyromonas levii. Canadian Journal of Veterinary Research* **63:** 113-118.

72. Lu, Y.-C., W.-C. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. *Cytokine* **42:** 145-151.

73. Madsen, J. S., M. Burmolle, L. H. Hansen, and S. J. Sorensen. 2012. The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology* **65:** 183-195.

74. Mah, T. F. C., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9:** 34-39.

75. Miao, L., Y. Liu, Q. Li, Z. Wang, H. Li, and G. Zhang. 2010. Screening and sequence analysis of the hemolysin gene of *Fusobacterium necrophorum*. *Anaerobe* **16:** 402-404.

76. Mills, S. W., S. H. Montgomery, and D. W. Morck. 2006. Evaluation of the effects of short-chain fatty acids and extracellular pH on bovine neutrophil function in vitro. *Am J Vet Res* **67:** 1901-1907.

77. Morck, D. W., K. Lam, S. G. McKay, M. E. Olson, B. Prosser, B. D. Ellis, R. Cleeland, and J. W. Costerton. 1994. Comparative evaluation of fleroxacin, ampicillin, trimethoprim-sulfamethoxazole, and gentamicin as treatments of catheter-associated urinary tract infection in a rabbit model. *International Journal of Antimicrobial Agents* **4:** S21-S27.

78. Morrison, D. C., and D. M. Jacobs. 1976. Binding of polymyxin B to lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* **13:** 813-818.

79. Nagaraja, T. G., S. K. Narayanan, G. C. Stewart, and M. M. Chengappa. 2005. *Fusobacterium necrophorum* infections in animals: pathogenesis and pathogenic mechanisms. *Anaerobe* **11:** 239-246.

80. Narayanan, S., G. C. Stewart, M. M. Chengappa, L. Willard, W. Shuman, M. Wilkerson, and T. G. Nagaraja. 2002. *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. *Infect Immun* **70:** 4609-4620.

81. Oscarsson, J., M. Karched, B. Thay, C. Chen, and S. Asikainen. 2008. Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. *BMC Microbiology* **8:** 206-219.

82. Park, B. S., D. H. Song, H. M. Kim, B. S. Choi, H. Lee, and J.O. Lee. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* **458:** 1191.

83. Periasamy, S., and P. E. Kolenbrander. 2009. Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *J Bacteriol* **191:** 6804-6811.

84. Permpanich, P., M. J. Kowolik, and D. M. Galli. 2006. Resistance of fluorescent-labelled *Actinobacillus actinomycetemcomitans* strains to phagocytosis and killing by human neutrophils. *Cell Microbiol* **8:** 72-84.

85. Peyyala, R., S. S. Kirakodu, J. L. Ebersole, and K. F. Novak. 2011. Novel model for multispecies biofilms that uses rigid gas-permeable lenses. *Appl Environ Microbiol* **77:** 3413-3421.

86. Poltorak, A., X. L. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science* **282:** 2085-2088.

87. Priester, J. H., A. M. Horst, L. C. Van De Werfhorst, J. L. Saleta, L. A. K. Mertes, and P. A. Holden. 2007. Enhanced visualization of microbial biofilms by staining and environmental scanning electron microscopy. *Journal of Microbiological Methods* **68:** 577-587.

88. Quade, M. J., and J. A. Roth. 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology and Immunopathology* **58:** 239-248.

89. Ramirez, S., T. G. Hild, C. N. Rudolph, J. R. Sty, S. C. Kehl, P. Havens, K. Henrickson, and M. J. Chusid. 2003. Increased diagnosis of Lemierre syndrome and other *Fusobacterium necrophorum* infections at a children's hospital. *Pediatrics* **112:** E380-E385.

90. Rastogi, R. P., S. P. Singh, D. P. Hader, and R. P. Sinha. 2010. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2 ',7 '-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937. *Biochemical and Biophysical Research Communications* **397:** 603-607.

91. Ray, A., M. Cot, G. Puzo, M. Gilleron, and J. Nigou. 2013. Bacterial cell wall macroamphiphiles: Pathogen-microbe-associated molecular patterns detected by mammalian innate immune system. *Biochimie* **95:** 33-42.

92. Rigby, K. M., and F. R. DeLeo. 2012. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Seminars in Immunopathology* **34:** 237-259.

93. Roos, D., R. van Bruggen, and C. Meischl. 2003. Oxidative killing of microbes by neutrophils. *Microbes and Infection* 5: 1307-1315.

94. Sabroe, I., L. R. Prince, E. C. Jones, M. J. Horsburgh, S. J. Foster, S. N. Vogel, S. K. Dower, and M. K. B. Whyte. 2003. Selective roles for toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *Journal of Immunology* **170:** 5268-5275.

95. Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology* **184:** 1140-1154.

96. Schletter, J., H. Heine, A. J. Ulmer, and E. T. Rietschel. 1995. Molecular mechanisms of endotoxin activity. *Archives of Microbiology* **164:** 383-389.

97. Schmitt, B. H., S. A. Cunningham, A. L. Dailey, D. R. Gustafson, and R. Patel. 2013. Identification of Anaerobic Bacteria by Bruker Biotyper Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry with On-Plate Formic Acid Preparation. *Journal of Clinical Microbiology* **51:** 782-786.

98. Shimoyama, T., S. Fukuda, Q. Liu, S. Nakaji, Y. Fukuda, and K. Sugawara. 2003. *Helicobacter pylori* water soluble surface proteins prime human neutrophils for enhanced production of reactive oxygen species and stimulate chemokine production. *Journal of Clinical Pathology* **56:** 348-351.

99. Sigge, A., A. Essig, B. Wirths, K. Fickweiler, N. Kaestner, N. Wellinghausen, and S. Poppert. 2007. Rapid identification of *Fusobacterium nucleatum* and *Fusobacterium necrophorum* by fluorescence *in situ* hybridization. *Diagnostic Microbiology Infect Dis* **58:** 255-259.

100. Smalley, J. W., A. J. Birss, and J. Silver. 2000. The periodontal pathogen *Porphyromonas gingivalis* harnesses the chemistry of the mu-oxo bishaem of iron protoporphyrin IX to protect against hydrogen peroxide. *FEMS Microbiology Letters* **183:** 159-164.

101. Stewart, P. S. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy* **40:** 2517-2522.

102. Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton. 2002. Biofilms as complex differentiated communities. *Annual Review of Microbiology* **56:** 187-209.

103. Summanen, P. H., B. Durmaz, M. L. Vaisanen, C. X. Liu, D. Molitoris, E. Eerola, I. M. Helander, and S. A. Finegold. 2005. *Porphyromonas somerae* sp nov., a pathogen isolated from humans and distinct from *Porphyromonas levii*. *Journal of Clinical Microbiology* **43:** 4455-4459.

104. Sweeney, J. F., P. K. Nguyen, G. M. Omann, and D. B. Hinshaw. 1997. Ultraviolet irradiation accelerates apoptosis in human polymorphonuclear leukocytes: protection by LPS and GM-CSF. *Journal of Leukocyte Biology* **62:** 517-523.

105. Swidsinski, A., Y. Dörffel, V. Loening-Baucke, F. Theissig, J. C. Rückert, M. Ismail, W. A. Rau, D. Gaschler, M. Weizenegger, S. Kühn, J. Schilling, and W. V. Dörffel. 2011. Acute appendicitis is characterised by local invasion with *Fusobacterium nucleatum/necrophorum*. *Gut* **60:** 34-40.

106. Tadepalli, S., S. K. Narayanan, G. C. Stewart, M. M. Chengappa, and T. G. Nagaraja. 2009. *Fusobacterium necrophorum:* a ruminal bacterium that invades liver to cause abscesses in cattle. *Anaerobe* **15:** 36-43.

107. Tan, Z. L., T. G. Nagaraja, and M. M. Chengappa. 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. *Vet Res Commun* **20:** 113-140.

108. Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, and J. S. Smith. 1994. Biological and biochemical characterization of *Fusobacterium nercophorum* leukotoxin. *American Journal of Veterinary Research* **55:** 515-521.

109. Tenson, T., M. Lovmar, and M. Ehrenberg. 2003. The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. *Journal of Molecular Biology* **330:** 1005-1014.

110. Tré-Hardy, M., C. Nagant, N. El Manssouri, F. Vanderbist, H. Traore, M. Vaneechoutte, and J. P. Dehaye. 2010. Efficacy of the combination of tobramycin and a macrolide in an in vitro *Pseudomonas aeruginosa* mature biofilm model. *Antimicrob Agents Chemother* **54:** 4409-4415.

111. Trent, M. S., C. M. Stead, A. X. Tran, and J. V. Hankins. 2006. Diversity of endotoxin and its impact on pathogenesis. *Journal of Endotoxin Research* **12:** 205-223.

112. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nature Reviews Immunology* **7:** 179-190.

113. Voyich, J. M., K. R. Braughton, D. E. Sturdevant, A. R. Whitney, B. Saïd-Salim, S. F. Porcella, R. D. Long, D. W. Dorward, D. J. Gardner, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo. 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *Journal of Immunology* **175:** 3907-3919.

114. Walter, M. R. V., and D. W. Morck. 2002. In vitro expression of tumor necrosis factor-alpha, interleukin 1 beta, and interleukin 8 mRNA by bovine macrophages following exposure to *Porphyromonas levii*. *Canadian Journal of Veterinary Research* **66:** 93-98.

115. Wang, G., Y. Gong, F. J. Burczynski, and B. B. Hasinoff. 2008. Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. *Free Radical Research* **42:** 435-441.

116. Wang, H., and J. A. Joseph. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine* **27:** 612-616.

117. White, David. 2007. Structure and Function, P.18,29-31. The Physiology and Biochemistry of Prokaryotes, 3rd edition. Oxford University Press, New York.

118. Wolcott, R., J. W. Costerton, D. Raoult, and S. J. Cutler. 2013. The polymicrobial nature of biofilm infection. *Clinical Microbiology and Infection* **19:** 107-112.

119. Wu, G. H., C. Jarstrand, and J. Nordenstrom. 1999. Phagocyte-induced lipid peroxidation of different intravenous fat emulsions and counteractive effect of vitamin E. *Nutrition* **15:** 359-364.

120. Yamamoto, C., S. Yoshida, H. Taniguchi, M. H. Qin, H. Miyamoto, and Y. Mizuguchi. 1993. Lipopolysaccharide and granulocyte colony-stimulating factor delay neutrophil apoptosis and ingestion by guinea pig macrophages. *Infection and Immunity* **61:** 1972-1979.

121. Yeom, J., Y. Lee, and W. Park. 2012. Effects of non-ionic solute stresses on biofilm formation and lipopolysaccharide production in *Escherichia coli* O157:H7. *Research in Microbiology* **163:** 258-267.