The Elucidation of the Biological Function of 3-Hydroxy Fatty Acids (3-OH C10:0) in the Pathogenesis of *Pseudomonas aeruginosa*

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TABLE OF CONTENT

Note: The thesis consists of different chapters, some in manuscript format. As a result, the repetition of some information could not be avoided.

TITLE PAGE

Title:	The Elucidation of the Biological Function of 3-Hydroxy Fatty Acids (3-OH C10:0) in the Pathogenesis of *Pseudomonas aeruginosa*
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Firstly, I thank God, for being my comfort, strength and wisdom. I completed my studies due to the presence of God in my life and the will to continue.

Ephesians 6: 10

"In conclusion, be strong in the Lord [draw your strength from him and be empowered through your union with him] and in the power of his [boundless] might"

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DECLARATION

I, Evodia Yolander Kgotle, hereby declare that the work presented in this thesis is my own independent investigations. In addition, I declare that this thesis has not been submitted, in full or part, to another institution of higher education for granting of PhD degree. The successful completion of the thesis has been made possible by a joint research grant from the National Research Foundation and the University of the Free State. There are no competing financial interests

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Candidate for a PhD degree

Friday, May 21, 2021

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ETHICAL CLEARANCE

The Biosafety and Environmental Ethics Research Committee, University of the Free State (UFS-ESD2019/0153/1504) approved the environmental and biosafety protocols.

The Animal Research Ethics Committee, University of the Free State (UFS-AED2017/0077), approved the animal experimental protocols.

THESIS

SUMMARY

CHAPTER LAYOUT

CHAPTER 1:

Literature Review

CHAPTER 2:

Investigating the production of 3-hydroxy fatty acids by *Pseudomonas aeruginosa*

CHAPTER 3:

Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vitro* studies

CHAPTER 4:

Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vivo* studies

CHAPTER 5:

General Discussion

THESIS

SUMMARY

3-Hydroxy fatty acids belong to a group of molecules called oxylipins, which are biological active molecules. Perhaps, the most well-known of these are the prostaglandins, including microbial-derived prostaglandins. In the past few years, there has been evidence documenting the importance of 3-hydroxy fatty acids to several organisms. To this end, the thesis has a few studies designed to contribute to a foundation of work targeted at implicating these molecules being possible pseudomonal virulence factors.

The thesis begins by interrogating literature concerning lipids and 3-hydroxy fatty acids for orientation purposes and to give the reader a deeper appreciation of these molecules. In Chapter 2 of the thesis, the sourced clinical pseudomonal isolates were first identified and subsequently, examined for the production of 3-hydroxy fatty acids. Through using commercial analytical standards, it was possible to detect the presence of several different 3-hydroxy fatty acids species. Importantly, one species, 3-hydroxydecanoic acid (3-OH C10:0), was produced at high enough concentrations, i.e. 1 µM per 10 million cells, that it was possible to design future studies. Based on the extraction protocol that was followed in the chapter, it was reasoned that 3-OH C10:0 was in a free-form. Nonetheless, the herein designed studies included a crude lipopolysaccharide (LPS) sample, as the LPS may be a source of pseudomonal 3-hydroxy fatty acids, for comparison reasons.

In Chapter 3, an *in vitro* study wherein a murine macrophage cell line was challenged with 3-OH C10:0 as designed. Macrophages are essential immune cells whose action can assist to resolve invading pathogens, and through antigen presentation and cytokine production – can harmonise and link innate and adaptive immunity. Similar

to the LPS, 3-OH C10:0 was shown to signal for production of the pro-inflammatory interferon-gamma (INF-γ) possibly by engaging a cellular programming that required the activation of the mitogen-activated protein kinase (MAPK) p38 pathway. In addition, 3-OH C10:0 impaired the uptake (internalisation or engulfment) of pseudomonal cells by macrophages possibly by suppressing the levels of fetuin A (FetA). Interestingly, the pseudomonal cells that were successfully taken up, seemed to survive the phagocytic event better in the presence of 3-OH C10:0 compared to in the absence of 3-OH C10:0. To explore this further, Chapter 4 set up experiments wherein whole, laboratory models (nematodes and rats) were challenged with 3-OH C10:0. First, the nematodes were shown to be affected in a number of ways. 3-OH C10:0 was shown to reduce the survival of these organisms, when compared to non-treated nematodes. Moreover, this molecule seemed to affect more the immunological response pathway when compared to the cellular development processes. Concerning the rats, 3-OH C10:0 led to increased levels of circulating monocytes, after 6 h of animal exposure. Based on the results of Chapters 3 and 4, it seems 3-OH C10:0 may be a virulence determinant, and this may be a relevant molecule for studying the immune response to pseudomonal infections.

Key words: 3-Hydroxydecanoic acid (3-OH C10:0), 3-Hydroxy fatty acids, Animal studies, *Caenorhabditis elegans* (nematodes), Inflammation, Interferon-gamma (INF-γ), Macrophages, MAPK pathway, *Pseudomonas aeruginosa*, *Rattus norvegicus* (rats), Signalling.

CHAPTER 1

Literature review

Manuscript based on this chapter has been prepared and will be submitted to the journal; Prostaglandins and Other Lipid Mediators for consideration.

1.1 ABSTRACT

Our limited knowledge concerning 3-hydroxy fatty acids, particularly those that are in free-form, stems out of studies conducted in fungi. These molecules are reported to influence a number of fungal processes, which include assisting in the liberation of spores from asci and acting as signal molecules, among others. However, there is evidence of the existence of similarly structured 3-hydroxy fatty acids in bacteria. In the thesis, special attention is given to pseudomonal 3-hydroxy fatty acids. Although the organism is considered an extracellular pathogen, it can also interact with host molecules and immune cells in the intracellular environment. Thus, it becomes important to understand how pseudomonal 3-hydroxy fatty acids may potentiate infectious processes. As a result, literature is interrogated in an attempt to understand how these molecules are produced, including how they are sensed in the extracellular environment and how they may influence certain pathologies, such as host-pathogen interactions. This information is relevant as it can reveal 3-hydroxy fatty acid biosynthetic pathways as targets for developing anti-virulence drugs. In turn, these drugs can be used in a complementary manner to buttress the action of traditional antibiotics.

Key words: 3-Hydroxy fatty acids, Bacteria, Fungi, Inflammation, Lipids, Signalling.

1.2 INTRODUCTION

Lipids are naturally occurring molecules that are found in all living organisms and display an amphiphilic quality when immersed in water (Kock *et al*., 1998). Based on their chemical structure, these molecules can be considered as simple (fatty acids) or complex molecules (due to the carbocation-based condensation of isoprene units) (Fahy *et al*., 2005). In some instances, lipids can complex with other macromolecules such as proteins or carbohydrates (Pompéia *et al*., 2000). For many years, lipids were described as energy stores that are often catabolised to provide maintenance energy in times of starvation (Houten and Wanders, 2010) or function as essential components of the cell membrane (Dennis, 2016). In the case of the latter, cholesterol assists in maintaining membrane fluidity (Alberts *et al*., 2002) while phospholipids found on mitochondrial membrane are critical in anchoring electron carriers (Goñi, 2014). In the next section, an attempt is made to highlight the relevance of lipids to other biological processes – beyond their traditional understanding as energy stores.

1.2.1 Lipids as inflammation markers and signalling molecules

Inflammation is part of the non-specific immune response response that is stimulated to clear the initial cause of cell injury, i.e. presence of pathogens (Ferrero-Miliani *et al*., 2007). Lipids are important mediators of inflammation. To illustrate this, lipids such as eicosanoids have been described to regulate the balance of inflammation during infection, by either causing inflammation or resolution of the inflammatory response (Dennis and Norris, 2015). Around 60 years ago, Bergström and co-workers worked out that arachidonic acid (AA) can be converted to prostaglandin E_2 (PGE_2) through a specific enzymatic reaction (Bergström *et al*., 1964). The molecule, PGE_2, was later

demonstrated to be important in inflammatory processes. More to this point, in his seminal work while assessing the pharmacological action of aspirin on a homogeneate guinea pig lung, John Vane came to the conclusion that aspirin inhibited the cyclooxygenase, an enzyme that is responsible for the formation of prostanoids, including thromboxane and prostaglandins (Vane, 1971). This enzyme (cycloxygenase) was shown to be in high concentration in endoplasmic reticulum of prostanoid-forming cells (Vane, 1971). In mammalian systems, cyclooxygenase (COX) enzymes are responsible for PGE_2 production. These enzymes catalyse the insertion of two oxygen atoms into arachidonic acid to form PGE_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Marnett *et al*., 1999; Murakami *et al*., 2003). Thereafter, PGE_2 inhibits T helper (Th) type 1 and promotes Th2 responses in mammalian hosts, inducing localized pro- or anti-inflammatory effects dependant on the host tissue affected (Romani, 2000). Takai and co-workers were the first to report that lipids can function as signalling molecules (Takai *et al*., 1979). The authors showed that diacylglycerol (DAG), was a critical molecule that effects signalling processes across the membrane by increasing the activity of protein kinase C. In turn, this may lead to cellular programming that regulates cell growth and mediation of immune responses (Heinisch and Rodicio, 2018).

Today, advances in molecular techniques have made it possible to design gene knockout studies – to better understand the role of lipids in cell signalling and inflammation. To illustrate this point, Pal et al. (2012) demonstrated the link between lipids and inflammation. In their study, they showed that the deletion of the fetuin A (FetA) gene (which serves as an endogenous ligand for the toll-like receptor (TLR) 4) in mice, led to a down-regulation of inflammatory signalling in adipose tissue. Fetuin

A is a major globulin component of serum that has been shown to regulate the function of macrophages (Wang *et al*., 1998). In some studies, this glycoprotein (FetA) has been reported to mediate the uptake of *Escherichia coli* and *Staphylococcus aureus* cells, including apoptotic cells, by phagocytes (van Oss *et al*. 1974; Jersmann *et al*., 2003). Interestingly, TLRs are embedded in a lipid rafts, which form part of the cell membrane structure (Simons and Ehehalt, 2002; Pike, 2003). To ensure normal signalling of the TLRs, the lipid rafts must be maintained at a balanced consistency, since cholesterol and polyunsaturated fatty acids can influence their function (Ruysschaert and Lonez, 2015; Varshney and Yadav, 2016). Thus, any impairment to the constitution of the lipid rafts may affect several signalling processes that are important to inflammatory diseases and microbial infections (Simons and Ehehalt, 2002; Varshney and Yadav, 2016). When considering the above, it is clear that lipids are more than just lipid stores. In the next sections, attention is given to 3-hydroxy fatty acids, and importantly their possible role in microbial pathogenesis.

1.3 3-HYDROXY FATTY ACIDS

3-Hydroxy fatty acids are oxygenated, lipid-based molecules that have a hydroxyl functional group on the beta carbon of the hydrocarbon chain (Kock *et al*., 2003, 2007; Sebolai *et al*., 2012). The hydrocarbon chain can be saturated or unsaturated and branched or unbranched (Fig. 1).

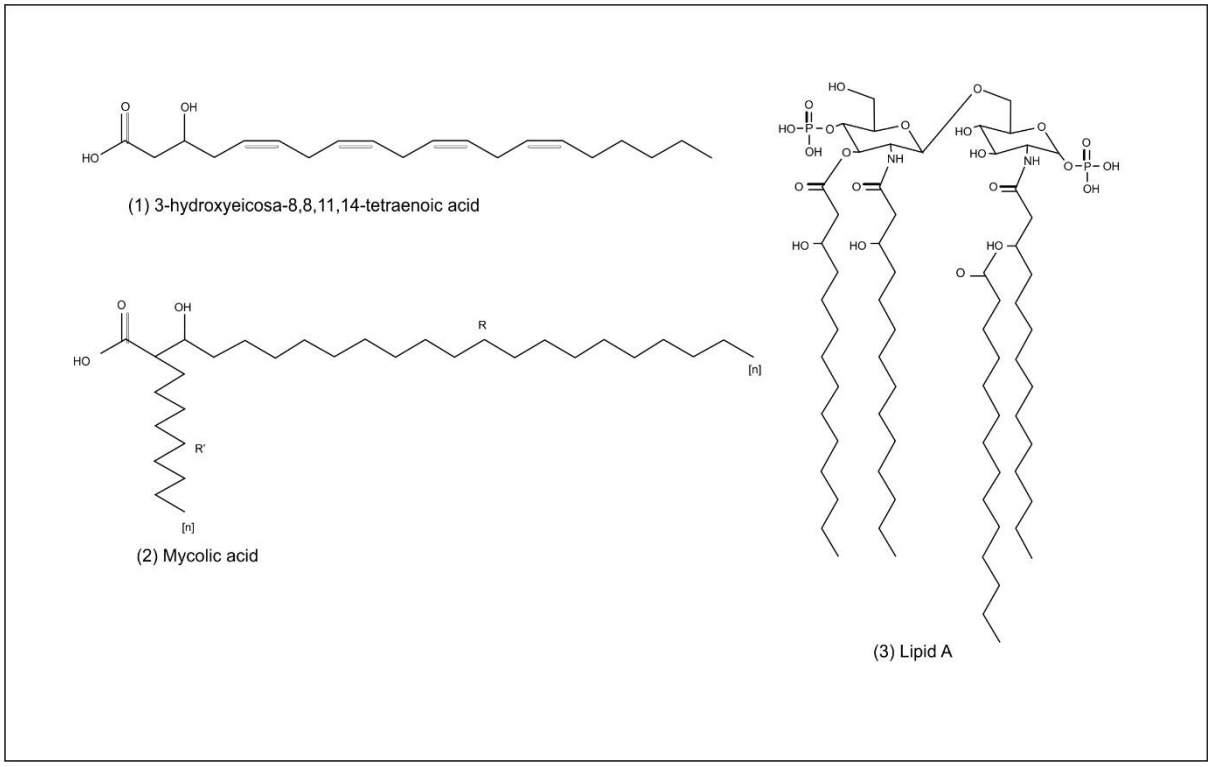


Fig 1. Chemical structures depicting various 3-hydroxy fatty acids. (1) This C-20 molecule is 3-hydroxy eicosatetraenoic acid (3-HETE) that may assume either (*R*) or (*S*) configuration. (2) A mycolic acid with 3-hydroxy fatty acid as a base molecule with elongated R′ and R, which in total, varies among organisms (Marrakchi et al 2014). The hydrocarbon chain may be straight or branched, saturated or unsaturated. (3) Lipid A, a complex glycolipid with O and N 3-OH fatty acids. The Lipid A is a component of the lipopolysaccharide layer.

These molecules are regarded as secondary metabolites with no apparent function in the primary metabolism of organisms (Kock *et al*., 2003, 2007; Tsitsigiannis and Keller, 2007). To illustrate this point, the presence of 3-hydroxy fatty acids in human blood or cells is considered unnatural and can have dire consequences. These molecules are secreted as a result of a defective mitochondrial long-chain hydroxy acyl-CoA dehydrogenase, possibly due to a mutation (Jones and Bennett, 2011). Unfortunately, the subsequent accumulation of 3-hydroxy fatty acids (due to a defect in the fatty acid

beta oxidation pathway) is reported to lead to the development of acute liver injury (Jones and Bennett, 2011). This condition is regarded as an obstetric and medical emergency in pregnant subjects. The same authors further suggested that these molecules can accumulate in hepatocytes, neurons, myocytes, cardiomyocytes and placental trophoblast cells, where they can lead to widespread damage by exerting lipo-toxicity, which is the uncoupling of mitochondrial oxidative phosphorylation, and diminished mitochondrial respiration.

However, in other organisms, 3-hydroxy fatty acids are purposely produced for a secondary function. For example, in birds, these molecules are secreted in the uropygial glands of female mallards, where they function as sex pheromones (Rajchard, 2007). In non-pathogenic fungi, they have been found coating surfaces of cell walls where they were implicated in effecting cell aggregation or participating in the release of spores from asci (Kock *et al*., 2000, 2003, 2007). Furthermore, in the non-pathogenic bacteria, *Lactobacillus plantarum*, these molecules act as anti-microbial agents that control the growth of spoilage yeasts and moulds during fermentation (Sjögren *et al*., 2003). Moreover, some Gram-negative bacteria may use the bacterial outer membrane vesicles to shed the lipopolysaccharide (LPS), also known as the endotoxin, to promote the secretion of pro-inflammatory cytokines (Raetz and Whitfield, 2002). The LPS is an important component of the outer membrane of Gram-negative bacteria that provides structural stability to the cell wall (Alexander and Rietschel, 2001). More importantly, in the context of this write up, the LPS has 3-hydroxy fatty acids as a constituent (Wilkinson, 1997; Miller *et al*., 2005). From the above, it is clear that these molecules have diverse functions and may present in different sources.

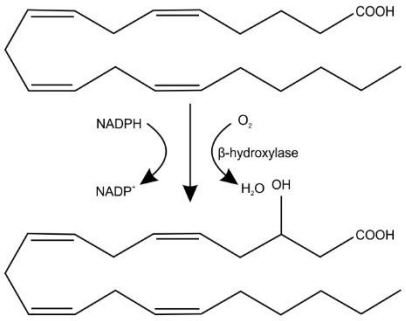
In the main, there are four proposed biosynthetic pathways that are reported to lead to the production of 3-hydroxy fatty acids (Fig. 2). These are:

(1) direct hydroxylation. Here, the beta-carbon on the hydrocarbon chain of a fatty acid molecule may be hydroxylated using a free oxygen molecule derived from the surrounding environment (Sebolai *et al*., 2012). The reaction is catalysed by the action of a monooxygenase such as the cytochrome P450 enzyme,

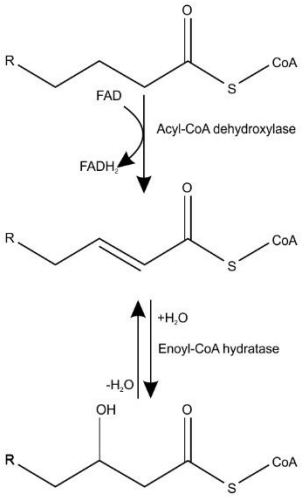
(2) Enzymatic reactions that constitute an incomplete mitochondrial beta oxidation process being the second one (Sebolai *et al*., 2012). Herein, the 3-D-hydroxyacyl-CoA metabolite is poorly metabolised by the mitochondrial enzyme, 3-hydroxyacyl-CoA dehydrogenase (Venter *et al*., 1997). Because of this, the D-enantiomer initially accumulates inside the mitochondria, and it is eventually excreted extracellularly as a 3-D hydroxy fatty acid (Sebolai *et al*., 2012),

(3) The fatty acid synthase (FAS) pathway, which involves the reduction of beta-ketoacyl-ACP to beta-hydroxyacyl-ACP by the NADPH-dependent beta-ketoacyl-ACP reductase (Hiltunen *et al*., 2005; Takayama *et al*., 2005; Martínez and Campos-Gómez, 2016), and

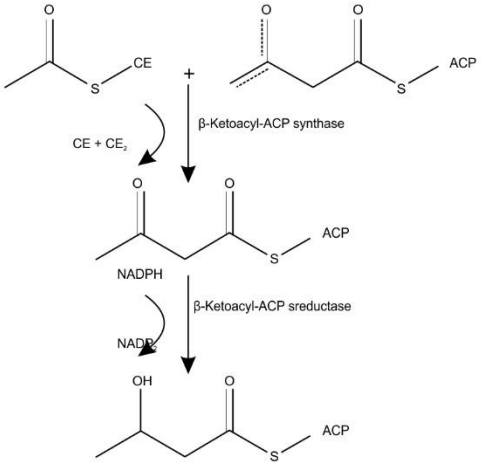
(4) 3-Hydroxy fatty acids may also be liberated from the lipopolysaccharide (LPS) of Gram-negative bacteria through the catalytic action of a lipid A-modifying enzyme; PagL (Geurtsen *et al*., 2005; Ernst *et al*., 2006; Boutrot and Zipfel, 2017; Kutschera *et al*., 2019). The enzyme removes 3-hydroxy fatty acids from the LPS by hydrolysing the ester bond at the 3 position of the hexa-acylated lipid A in the outer membrane.



(1) Direct hydroxylation pathway



(2) Mitochondria β-oxidation pathway



(3) Fatty acid synthase pathway

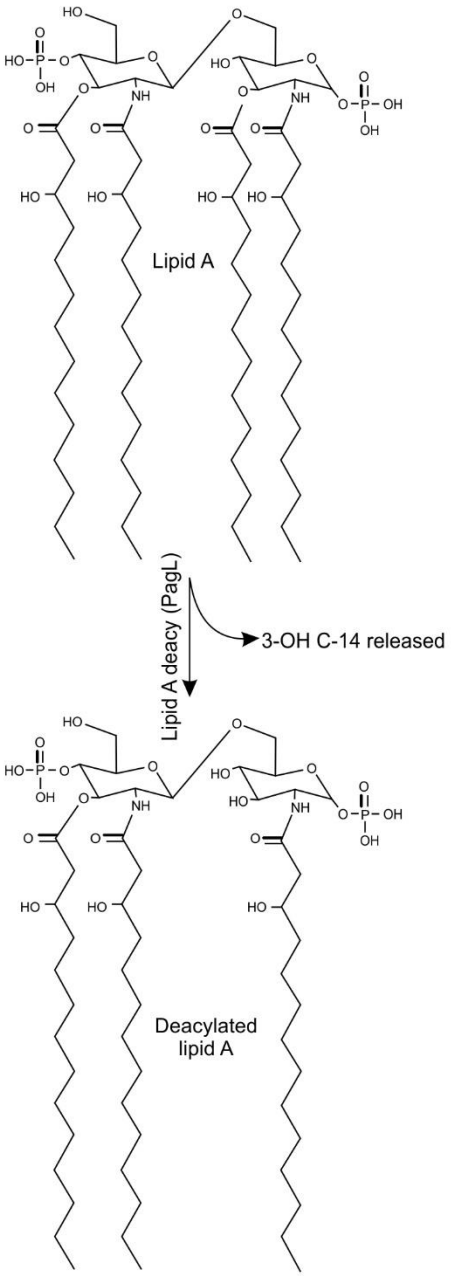


Fig 2. The different enzymatic biosynthetic pathways leading to the production of 3-hydroxy fatty acids. Image is modified from Sebolai et al. (2012).

1.3.1 Role of 3-hydroxy fatty acids in pathogenic microbes

As quorum sensing molecules

The plant pathogenic bacterium, *Ralstonia solanacearum*, through the action of a methyltransferase is reported to produce 3-hydroxy palmitate (Flaver *et al*., 1997) and 3-hydroxy myristate (Kai, 2018), which act as quorum sensing molecules. These fatty acids thus act as essential, early signalling molecules in a cascade that controls the expression of virulence (Flavier *et al*., 1997; Kai, 2018). Once the production of these 3-hydroxy fatty acids reaches a particular threshold, there is an increase in the production of extracellular polysaccharide (EPS) by the cells, which induces severe wilting in the infected plant by preventing water flow in the xylem (Flavier *et al*., 1997; Kai, 2018). Importantly, these molecules have been shown to be volatile, as a result Flavier argued that they can affect long-distance communication between spatially separated colonies (Flavier *et al*., 1997).

The human yeast pathogen, *Candida albicans*, has been shown to produce 3-hydroxy tetradecaenoic acid from the biotransformation of linolenic acid (Nigam *et al*., 2011). This 3-hydroxy fatty acid was reported to act as a quorum sensing molecule that can alter gene expression. At a particular cell density, the cells were reported to engage in a quorum sensing mechanism that allows for accelerated hyphal formation, which in turn, may lead to increased penetration of host tissue (Nigam *et al*., 2011).

As immuno-modulators

The Nigam group were the first to study the biological effects of microbial 3-hydroxy fatty acids in a host organism (Nigam *et al*., 1999). The authors showed that 3-hydroxy fatty acids affected signal transduction processes in human neutrophils and tumour cells in multiple ways, possibly via a G-protein receptor. Moreover, the study showed that 3-hydroxy fatty acids were a strong chemotactic agent. Ciccoli et al. (2005) reported that during the course of an infection, *Candida albicans* can scavenge AA from inflamed host cells and convert it to prostaglandins, in a reaction that is catalysed by the COX enzyme. Interestingly, the same authors showed that 3-hydroxy eicosatetraenoic acid (3-HETE), which is stereo-chemically identical to AA, could also be converted to prostaglandins by the COX enzyme. This was not surprising as their modelling of 3-HETE and COX enzyme molecular interaction revealed a similar enzyme-substrate structure as reported for AA and COX enzyme (Ciccoli *et al*., 2005). The oxygenation of 3-HETE by COX enzyme led to the production of 3-hydroxy PGE_2, which induced the expression of interleukin-6 in A549 cells and raised the levels of cyclic AMP in Jurkat cells (Ciccoli *et al*., 2005). A recent study by Kutschera et al. (2019) using *Arabidopsis* as a model, showed that bacterial 3-hydroxy fatty acids can also elicit an immune response in plants. Here, the 3-hydroxy fatty acids were sensed by the plant's lectin receptor kinase.

As anti-phagocytic molecules

Recent reports in fungal cells have suggested that 3-hydroxy fatty acids have an anti-phagocytic quality. Using amoeba as a model for macrophages, Madu et al. (2015) showed that 3-hydroxy fatty acids (3-OH C9:0) impaired the uptake of cryptococcal cells by amoeba. Moreover, internalised cryptococcal cells survived better in the

presence of 3-hydroxy fatty acids compared to in the absence. The above observations were attributed to the ability of 3-hydroxy fatty acids to suppress the levels of amoebal FetA-like protein which is a factor that promotes phagocytosis. In this regard, 3-hydroxy fatty acids could, in part, impair the intracellular signalling mechanism that is required to initiate phagocytosis. These molecules also shield cells against the effects of hydrogen peroxide and amoebapore; a hydrolytic enzyme that kills internalised cells (Madu *et al*., 2017). The idea of lipids may affect the anti-microbial environment of the phagosome has been documented elsewhere. For example, Eftimiadi et al. (1987) documented that fatty acids may prevent the release of lysozymes while Bellinati-Pires et al. (1993) reported that fatty acids may reduce hydrogen peroxide production.

1.3.2 3-Hydroxy fatty acids as constituents of other molecules

3-Hydroxy fatty acids can also be linked to other macromolecules. This is a quality more often seen in microbes. Examples where 3-hydroxy fatty acids form components of some macromolecules are discussed further.

Lipopolysaccharide of Gram-negative bacteria and fungi

The compositional analysis of the Gram-negative bacterial LPS show that it is constituted by a sugar moiety (galactosamine, glucosamine, galactose and glucose) and it is linked to a lipid A fraction that contains different 3-hydroxy fatty acids species (Rietschel *et al*., 1994). The LPS is found in the outer layer of non-encapsulated cells. Thus, it is exposed on the surface and may serve as an antigenic determinant. Several assays have been developed that target 3-hydroxy fatty acids in the lipid A (Lee *et al*.,

2004). The detected 3-hydroxy fatty acids are used as biomarkers to indirectly estimate the amount of present LPS (endotoxin), and in turn, this is correlated to the presence of Gram-negative bacteria in atmospheric bioaerosols (Lee *et al*., 2004).

The presence of the LPS in the blood could also initiate infectious processes (Rietschel *et al*., 1994; Brightbill and Modlin, 2000; Seydel *et al*., 2000; Alexander and Rietschel, 2001). For this reason, The LPS has been studied extensively. Part of these studies have asserted that the immunomodulatory centre of the LPS resides in the lipid A component (Rietschel *et al*., 1994; Seydel *et al*., 2000). More to this, using a plant (*Arabidopsis*) as a test host organism, Kutschera et al. (2019) showed that the depletion of 3-hydroxy fatty acids from bacterial LPS preparations failed to elicit an immune response in the plant.

The LPS has also been shown to be detrimental to many organisms. The presence of a Gram-negative cell or purified LPS in experimental animals is reported to cause several pathophysiological responses that may, in a worse case, lead to death of a susceptible host organism (Sampath, 2018). The presence of Gram-negative cells in a host evokes innate immune cells to engage in phagocytosis (Woolard and Frelinger, 2008). Here, the LPS may be sensed via the CD14/TLR4/MD2 receptor complex by innate cells (Park and Lee, 2013), which then initiates the production of proinflammatory cytokines (Dinarello, 2000; Annane *et al*., 2005). When the LPS is shed, a host organism may seek to neutralise the LPS either by enzymatic degradation or by complement-mediated detoxification, while insects may use immuno-proteins found in the haemolymph to bind the LPS (Sampath, 2018).

Interestingly, although the LPS is a virulence determinant that is primarily found in Gram-negative bacteria, it has also been detected in fungi. Cheng et al. (2005) showed that the medically important fungus, *Antrodia camphorata* can produce LPS with slight differences. For example, the sugar compositional analysis showed that sorbitol was present in the fungal neutral sugars but, absent in bacterial neutral sugars. Concerning the biological activity, the fungal LPS displayed lower cytotoxicity towards endothelial cells than bacterial LPS (Cheng *et al*., 2005). Furthermore, the fungal LPS was shown to differentially reverse the bacterial LPS-induced intercellular adhesion molecule-1 and monocyte adhesion. Based on their findings, the authors reasoned that the fungal LPS was anti-inflammatory, and there is scope for it to be administered to resolve the pro-inflammatory quality of bacterial LPS.

Mycolic acids of *Mycobacterium*

The mycolic acids are specific lipid components that form part of the mycobacterial cell envelope (Barry *et al.,* 1998; Dubnau *et al*., 2000; Marrakchi *et al*., 2014). This permeability barrier is made up of long α-alkyl side chains that are connected to 3-hydroxy fatty acids, which can either be synthesised by type I (FAS-I) or type II (FAS-II) fatty acid synthetase (Takayama *et al*., 2005). This cell structure is important to the survival of the mycobacterial cells as it can impair the action of antibiotics through cell wall coating (De Souza *et al*., 2008; Marrakchi *et al*., 2014). This 3-hydroxy fatty acid-rich structure also aids mycobacterial cells to grow in harsh conditions such as the often-deadly degradative environment that prevails inside macrophages (Cambier *et al*., 2014; Queiroz and Riley, 2017). As such, cells can escape immuno-processing.

Similar to the LPS, mycolic acids can also elicit an immune response (Cambier *et al*., 2014). The release of mycolic acids from the cell wall is reported to lead to the up regulation of the immune response (Fenton, 1998; Gordon, 2002). It is hypothesised that most of the lung damage that is sustained during tuberculosis is due to the up regulation of the inflammatory response (Ravimohan *et al*., 2017).

1.4 *PSEUDOMONAS AERUGINOSA* AND ITS PATHOGENESIS

Some of the studies that are presented herein were designed with the objective of implicating the lipid-based 3-hydroxy fatty acids in the pathogenesis of *Pseudomonas aeruginosa*. For this reason, a brief discussion on *Pseudomonas* is presented in order to place the organism in context of these studies.

Pseudomonas aeruginosa is a Gram-negative bacterium that has the following defining characteristics: rod morphology, aerobic, flagellated and non-spore forming (Morrison and Wenzel, 1984; van Delden and Iglewski, 1998). The organism can also secrete exopolysaccharides such as alginate that contribute to biofilm formation (Høiby *et al*., 2010). Biofilms are important to *P. aeruginosa* as they can promote surface colonisation (Gellatly and Hancock, 2013). This bacterium can also inhabit a variety of niches *viz*. ranging from aquatic to terrestrial environments, including eukaryotic organisms (Pier, 1985; Tan *et al*., 1999). Because of the latter, this bacterium is increasingly recognised as an opportunistic pathogen that can cause life-threatening infections in immunocompromised host (Govan and Deretic, 1996; Tan *et al*., 1999; Kerr and Snelling, 2009; Mittal *et al*., 2009; Høiby *et al*., 2010; Gellatly and Hancock, 2013). Thus, *P. aeruginosa* is of clinical importance.

Pseudomonas aeruginosa cells typically infect the upper respiratory tract, wounds and in some instances, cause bacteraemia (Lyczak *et al*., 2000; van Delden, 2007; Rella *et al*., 2012). The organism is often observed in nosocomial settings colonising medical devices (Kerr and Snelling, 2009). Pseudomonal infections are also observed in patients with cystic fibrosis (CF) (Høiby *et al*., 2010). Cystic fibrosis is an autosomal recessive disorder that results in a bronchial mucus that can promote the growth of pseudomonal cells (Delhaes *et al*., 2012). The disorder is caused by a mutation that exists in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Andersen, 1938; Delhaes *et al*., 2012). It has proven difficult to eradicate *P. aeruginosa* infection, primarily due to high levels of innate antibiotic resistance (difficult to phagocytose biofilms) and ever-increasing incidences caused by multidrug resistance strains of this bacterium (de Kievit *et al*., 2001; Fisher *et al*., 2005). It is, therefore, not surprising that *Pseudomonas aeruginosa* is a major cause of morbidity and mortality (Kerr and Snelling, 2009).

Pseudomonas aeruginosa possesses a number of virulence factors that are relevant to the pathogenesis of clinical isolates. Some of these factors are trafficked extracellularly via a wide range of secretory systems (Filloux, 2012). For example, exotoxin A is released by a type II secretion system (Hogardt *et al*., 2004). The toxin inactivates the eukaryotic elongation factor 2, which aids cells to synthesise proteins and necrotise (Kipnis *et al*., 2006). On the other hand, the exoenzyme U is secreted into host cells via a type III secretion system (Hogardt *et al*., 2004; Kipnis *et al*., 2006). Upon release, the enzyme degrades the host cytoplasmic membrane leading to lysis (Kipnis *et al*., 2006). In addition to these factors, the organism also possesses other important virulence factors such the lipid based rhamnolipids (Kipnis *et al*., 2006; Mittal

et al., 2009). Rhamnolipids are unique glycolipids that were originally discovered in *Pseudomonas aeruginosa*, however, they have been subsequently shown to be produced by other bacterial species (Abdel-Mawgoud *et al*., 2010). Rhamnolipids play a role in swarming motility of *P. aeruginosa* and has been implicated as playing part in the development of ventilator associated pneumonia (VAP) (Köhler *et al*., 2010).The molecule is made up of a rhamnose sugar that is linked to a 3-hydroxy fatty acid through a beta-glycosidic bond (Soberón-Chávez *et al*., 2005). Rhamnolipids have been identified to have antimicrobial properties that function by intercalating into the biological membrane of other microbes, causing loss of membrane integrity (Sotirova *et al*., 2008). In a study by Zulianello and co-workers, rhamnolipids were reported to be essential for the initial alteration of the epithelial barrier, which then allows bacteria to invade a host. Here, these molecules incorporate themselves into the epithelial cell membrane, inducing a decrease in the transepithelial resistance and the permeability of epithelial cells. This action compromises the epithelial barrier by widening tight junctions between host epithelial cells, which then allows *P. aeruginosa* cells passage into the paracellular pathway (Zulianello *et al*., 2006). Moreover, these molecules have been shown to inhibit and kill epithelial cells as well as inhibit macrophage phagocytosis (McClure and Schiller, 1992; 1996; Jensen *et al*., 2007). It has been reported that *in vitro* incubation of human monocyte-derived macrophages with rhamnolipids induced structural alterations in the macrophage cell membrane, which inhibited the phagocytosis of *Staphylococcus epidermidis* (McClure and Schiller, 1992). In a study by Kharazmi and co-workers, it was observed that purified rhamnolipids were able to induce direct neutrophil chemotactic activity and the oxidative burst response of monocytes was also enhanced by preincubation with this glycolipid (Kharazmi *et al*., 1989). Furthermore, rhamnolipids can stimulate the release

of interleukin (IL)-8, granulocyte-macrophage colony-stimulating factor, and IL-6 from nasal epithelial cells at non-cytotoxic levels (Bédard *et al*., 1993). Interestingly, these molecules have also been observed to facilitate the surface-associated migration of bacteria in a biofilm and, therefore, the initial microcolony formation and differentiation of the biofilm structure (Pamp and Tolker-Nielsen, 2007). Information from these studies suggest that rhamnolipids may contribute to the inflammatory-related tissue damage observed in lungs of cystic fibrosis patient. In part, this ability may also be attributed to the biosurfactant quality of this glycolipid, which assists the pathogen in lung surfactant solubilisation leading to tissue invasion (Rahim *et al*., 2001). *P. aeruginosa* has also been shown to produce free-form fatty acids that have a secondary function. More to this, cells have been reported to produce hydroxy fatty acids, *i.e.* 7, 10-dihydroxy-8-(*E*)-octadecenoic acid (Hou, 2008, Fourie, 2016). This compound is documented to display antimicrobial activity against *Bacillus subtilis* and *Candida albicans*. The organism also produces 15-hydroxy eicosatetraenoic acid (15-HETE), which is similar to mammalian 15-HETE (Vance *et al*., 2004). Serhan (2002) suggested that these molecules may be used to produce other eicosanoids, and, in turn, promote disease progression.

The above paragraph shows that pseudomonal cells can produce lipid-based virulence factors. And, by necessarily implication, it is possible that they may also secrete 3-hydroxy fatty acids that may act as virulence factors.

1.5 CONCLUSIONS

3-Hydroxy fatty acids are oxygenated fatty acids that are ubiquitous in nature, and thus perform diverse functions in different organisms. Our knowledge of these molecules, particularly when found in a free-form, suggests that they are involved in fungal cellular processes such as growth and development. There is evidence that these molecules may also influence host-pathogen interactions and possibly potentiate infectious processes. Host studies by Nigam et al. (1999) and Kutschera et al. (2019) have revealed the importance of G-protein coupled receptors in the sensing of 3-hydroxy fatty acids. To date, the Kutschera et al. (2019) study was the first that examined the response of a plant to bacterial 3-hydroxy fatty acids. Therefore, there is scope to study how a mammalian host may also respond to bacterial 3-hydroxy fatty acids. This information could shed light on immunological processes that are elicited by bacterial 3-hydroxy fatty acids during the course of an infection. More importantly, it may reveal information related to targets for drug development. To emphasise this point, the Madu et al. (2015) study showed the *in vitro* co-cultivation of cryptococcal cells (shown to secrete 3-hydroxy fatty acids) with phagocytic cells in the presence of aspirin and the co-cultivation of cryptococcal cells (that cannot produce 3-hydroxy fatty acids) with phagocytic cells in the presences of exogenously added 3-hydroxy fatty acids and aspirin. This study demonstrated that the presence of aspirin made cells susceptible to phagocytosis (Madu *et al*., 2015). This was due to aspirin being a competitive inhibitor for mitochondrial beta-oxidation enzymes (aspirin has structural similarities with intermediate products of beta-oxidation) that led to 3-hydroxy fatty acid production. However, aspirin has undesired side effects. Therefore, there is also scope to find other suitable anti-3-hydroxy fatty acids drugs.

1.6 AIMS OF THE THESIS

The thesis is not structured in a classical way; and as such, it is composed of three research chapters (Chapters 2, 3 and 4), which are in publication format. To this end, repetition of some information in the literature review could not be avoided. A general discussion and recommendation section (Chapter 5) is also included.

The overall aim was to elucidate the 3-hydroxy fatty acids, 3-OH C10:0, in the pathogenesis of *Pseudomonas aeruginosa*. The specific aim of each research chapter is listed below:

Chapter 2: Investigating the production of 3-hydroxy fatty acids by *Pseudomonas aeruginosa*.

Objectives:

2.1: To identify the obtained pseudomonal clinical isolates used in the study

2.2: To detect and quantify the presence 3-hydroxy fatty acids in the clinical isolates

Chapter 3: Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vitro* studies.

Objectives:

3.1: To evaluate the effects of 3-hydroxydecanoic acid (3-OH C10:0; shown to be produced by pseudomonal cells) on a macrophage murine cell line.

Chapter 4: Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vivo* studies.

Objectives:

4.1: To evaluate the effects of 3-OH C10:0 (shown to be produced by pseudomonal cells) on *Caenorhabditis elegans* (nematodes)

4.2: To evaluate the effects of 3-OH C10:0 (shown to be produced by pseudomonal cells) on *Rattus norvegicus* (rats)

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CHAPTER 2

Investigating the production of 3-hydroxy fatty acids by

Pseudomonas aeruginosa

Manuscript based on this chapter has been prepared and will be submitted to the Journal of Lipid Research for consideration.

2.1 ABSTRACT

The current chapter reports on the production of 3-hydroxy fatty acids by clinical isolates of *Pseudomonas aeruginosa*. These isolates were first identified using a polymerase chain reaction (PCR) with primers specific for bacteria as well as detection of pyocyanin production. Through using High Performance liquid-chromatography mass spectrometry (HPLC-MS), it was determined that the isolates produced a number of 3-OH fatty acid species at varying concentrations. Most notably, a pseudomonal isolate that was designated as *P. aeruginosa* LMPE 310 was able to produce 1 µM of the species 3-hydroxydecanoic acid, at a cell density of 1 x 10^8 cells/ml. This is a concentration that could be prepared in a laboratory setting when attempting to determine the biological function of this 3-hydroxy fatty acid species.

Keywords: 3-Hydroxy fatty acids, Clinical isolate, High performance liquid chromatography mass spectrometry, Polymerase chain reaction, *Pseudomonas aeruginosa*, Pyocyanin.

2.2 INTRODUCTION

Pseudomonas aeruginosa is a rod-shaped Gram-negative bacteria that is ubiquitous in nature (Rahme *et al*., 1997; Atzél *et al*., 2008). The organism is non-spore forming, catalase positive and cells can also oxidise glucose in oxidation/fermentation test using the Hugh and Leifson test (Krieg, 1984; Atzél *et al*., 2008). Although the organism has been regarded as a strict aerobe, it can also grow in low oxygen atmosphere – this, allows the organism to colonise many habitats, including artificial environments (Krieg, 1984; Gellatly and Hancock, 2013). The organism can also secrete siderophore such as pyocyanin, which is an antimicrobial toxin. *Pseudomonas aeruginosa* is of medical importance as if often found in nosocomial settings, where it can infect patients to cause severe sepsis, urinary tract, and respiratory tract, among others (Sadikot *et al*., 2005; Kerr and Snelling, 2009; Gellatly and Hancock, 2013). The pathogenesis of this pathogen is mediated by a wide range of virulence factors that assist in the maintenance of chronic infections (Kerr and Snelling, 2009).

Of interest is the documented texts that suggest that *Pseudomonas* can use lipids as virulence determinants. More to this, pseudomonal cells have been documented to scavenge arachidonic acid and convert it into a hydroxy fatty acid, *viz*. 15-hydroxy eicosatetraenoic acid (15-HETE), via the action of Lipoxygenase A (LoxA), which is a bacterial lipoxygenase (Vance *et al*., 2004). Lipoxygenases typically catalyse the abstraction of hydrogen and insertion of oxygen at specific fatty acid carbon-carbon double bonds to form hydroperoxides that can be reduced to alcohols or further transformed to potent mediators that may promote disease progression (Vance *et al*., 2004).

Hydroxy fatty acids are varied and present in many forms. For example, arachidonic acid, has also been shown to be bio-transformed into aspirin-sensitive 3-hydroxy eicosatetraenoic acid (3-HETE) by the fungus *Dipodascopsis uninucleata* (van Dyk *et al*., 1991) and into 3-hydroxy-5,8-tetradecadienoic acid (3-HDTE) by *Mucur genevesis* (Pohl *et al*., 1998). The latter expands on the nature of bioactive hydroxy fatty acids that may be produced by microbes to include 3-hydroxy fatty acids. However, their key feature is the characteristic hydroxyl group that is positioned on the beta-carbon.

The presence of 3-hydroxy fatty acids (in a free-form and not attached to other macromolecules) has been extensively reported in yeasts. However, in bacteria, there is limited information on 3-hydroxy fatty acids in a free-form. A study by Sjögren et al. (2003) show that the Gram-positive bacteria *Lactobacillus plantarum* secretes several 3-hydroxy fatty acids (that are in a free-form) as a defence mechanism against spoilage yeasts and moulds (Sjögren *et al*., 2003). Moreover, the plant pathogen, *Ralstonia solanacearum*, also secretes 3-hydroxy fatty acids (3-hydroxypalmitic methyl ester) that signals for the expression of virulence factors, such as the production of extracellular polysaccharides and endoglucanase (Flavier *et al*., 1997). There is also information on 3-hydroxy fatty acids when attached to other moieties that are located in the bacterial cell wall. For example, the bacterial lipopolysaccharide (LPS; endotoxin) layer contains a lipid A fraction, which has 3-hydroxy fatty acids as a constituent (Rietschel *et al*., 1994). This sepsis-causing endotoxin (Alexander and Rietschel, 2001) is reported to be responsible for the toxic immuno-modulating properties of the LPS (Rietschel *et al*., 1994; Barry *et al*., 1998; Seydel *et al*., 2000; Alexander and Rietschel, 2001). 3-Hydroxy fatty acids can also be found esterifying

sugar units to constitute the mycolic acids of *Mycobacterium* species (Barry *et al*., 1998; Takayama *et al*., 2005).

Against this background, the current study is bio-prospective and seeks to determine the presence of 3-hydroxy fatty acids, particularly those in a free-form, in the tested *Pseudomonas aeruginosa* clinical isolates. Based on the extraction protocol that was followed, it was theorised that 3-hydroxy fatty acids will be detected in a free-form. However, it is also conceivable that these molecules may be mobilised from macromolecules such as the LPS and rhamnolipids during extraction. In the LPS, the 3-hydroxy fatty acids are situated at the lipid A segment of the molecule, where it can either be *N*- and *O*-linked to the diglucosamine bisphosphate backbone (Knirel *et al.,* 2006; Pier, 2007). While in the rhamnolipids, the 3-hydroxy fatty acids are linked to either a mono- or di-rhamnose sugar moiety through a beta-glycosidic bond to form a mono-rhamnolipids or a di-rhamnolipids (Soberón-Chávez *et al*., 2005).

2.3 MATERIALS AND METHODS

2.3.1 Cultivation and standardisation of cells

Ten clinical *P. aeruginosa* isolates and a reference *P. aeruginosa* strain viz PA01, were used in the study. The clinical isolates were obtained from patients at the Universitas Academic Hospital (Bloemfontein, South Africa) where they were initially identified at the hospital using biochemical tests. Upon receipt, the isolates were sequentially assigned the following strain names: *P. aeruginosa* LMPE 302, *P. aeruginosa* LMPE 303, *P. aeruginosa* LMPE 304, *P. aeruginosa* LMPE 305, *P. aeruginosa* LMPE 306, *P. aeruginosa* LMPE 307, *P. aeruginosa* LMPE 308, *P.*

aeruginosa LMPE 309, *P. aeruginosa* LMPE 310, and *P. aeruginosa* LMPE 311. These isolates are preserved in glycerol as stocks for long term storage and are kept at the University of the Free State.

Pure cultures of all the isolates were separately streaked out on nutrient agar (23 g/l, Sigma-Aldrich, United States) at 37 ºC for 24 h. A loopful of the cells (from the 24 h agar plate) was used to inoculate a 50 ml centrifuge tube (Becton-Dickinson Labware, United States) that contained 25 ml of nutrient broth (25 g/l, Sigma-Aldrich, United States). The inoculated tubes were incubated overnight at 37 ºC while shaking at 160 rpm on a shaker. For standardisation of the cells, the optical density (OD) of each culture was first measured at OD_{600nm} using WTW PhotoLab S6 (LABTEC, United States) photometer. The obtained reading was substituted into the equation; cells/ml = OD_{600} x 2.5 x 10^8, by Jacobsen et al. (2011) to determine the number of colony-forming units per millilitre (cells/ml) (Jacobsen *et al*., 2011). With this equation, the cell concentration of pseudomonal cells was adjusted using fresh nutrient broth to reach a final cell concentration of 1 x 10^6 cells/ml. This was the initial determined cell density prior to any further incubation or experimental work.

2.3.2 Confirmation of the clinical isolates' identity

2.3.2.1 Polymerase chain reaction (PCR) and sequencing

The isolates were transported from the hospital in a box at room temperature, 48 h after being sampled. As isolates were obtained on plates from the hospital, it was important to confirm their identity. The thermal shock method was used for DNA extraction (Motaung *et al*., 2012). In brief, the DNA was extracted from 24 h old cultures that were grown in nutrient broth. In sterile PCR tubes, 5 µl of the cell culture

was added to 20 µl PCR-grade water, and the suspension was heated at 94ºC for 10 min to lyse the cells then stored at -20 ºC. The PCR identification (16S rRNA) was performed according to the method of Spilker et al. (2004) with slight modifications. The PCR reaction was performed using the KAPA *Taq* PCR kit (KAPA Biosystems) in 50 µl total reaction mixtures. To the point, 25 µl of the DNA suspension was added to the 25 µl of a double-concentrated reaction mixture containing 0.2 µl KAPA *Taq* polymerase, 1 µl of 10 mM deoxynucleotide triphosphate, 5 µl PCR buffer (A), 1 µl of 100 pm/ml oligonucleotide primers (forward and reverse) and 16.8 µl distilled water. An Applied Biosystems thermal cycler (model 2720) was used for the amplification of the DNA, with the total time of amplification being approximately 2 h. The cycle was programmed as follows: a denaturation step for 5 min at 94ºC followed by 30 amplification cycles, each consisting of 30 sec at 94ºC, and a 30 sec annealing step at 50ºC with 2 min extension at 72ºC. A final extension of 10 min at 72ºC was applied with cooling at 4ºC for 1 sec. The universal primer set (27f primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1472r primer: 5'-TACCTTGTTACGACTT-3') was used to amplify the 16S ribosomal DNA sequence of the clinical isolates. The PCR products were visualised on a 0.8% agarose gel (Agarose Di LE, Whitehead Scientific, South Africa). The SyBR® (Invitrogen™, United States) safe DNA stain was used to confirm the sizes of the amplicons.

The amplicons were cleaned-up before sequencing according to the method of Werle et al. (1994). In brief, 5 µl of the PCR products were mixed with 0.5 µl (10 U) exonuclease I (Exo I) (Thermo Scientific, United States) and 1 µl (1 U) FastAP™ thermosensitive alkaline phosphatase (Thermo Scientific, United States). The mixture was thereafter incubated at 37 ºC for 15 min. The reaction was then stopped by

heating the mixture at 85ºC for 15 min. A comparative 16S rDNA sequence analysis was performed utilising a Sanger sequencing run to confirm PCR-based identification results using the DNA sequencing BigDye terminator v. 3.1 kit (Applied Biosystems, United States). Resultant sequences were visualised as chromatograms and manually edited using Geneious version R10 (Biomatters Ltd, New Zealand; http://www.geneious.com), and the consensus sequences were identified using the basic local alignment search tool (BLAST) and compared to sequences currently available in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). The procedure was repeated for all isolates.

2.3.2.2 Pyocyanin production

After cultivating the cells as mentioned in 2.3.1, the broth revealed a bluish-green shade that was observable following overnight incubation. Based on this observation, the isolates were tested for the production of pyocyanin using the Essar et al. (1990) method. Before extraction, the cell density was determined to be 1 x 10^8 cells/ml. Following this, a 5 ml culture sample was transferred to a glass test tube. Extraction was achieved by adding a 5 ml chloroform solution (Merck) to the test tubes. This was followed by a re-extraction step using a 1.5 ml solution of 0.2 M hydrochloric acid (Merck). The top, chloroform fraction (has a slight pink colour) was targeted. A 200 µl solution of this fraction was aspirated and dispensed into a 96-wells sterile microtiter plate (Thermo Scientific). The absorbance of the plate was measured at 520 nm using spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom). The pyocyanin concentrations were then calculated by multiplying the optical density (OD_{520nm}) with a factor of 17.072 (Essar *et al*., 1990). In the end, the concentrations were expressed as µg/ml of pyocyanin produced per 1 x 10^8 cells/ml. The procedure

was repeated for all isolates. Three independent experiments were conducted for each isolate.

2.3.3 3-Hydroxy fatty acids: extraction and analysis

An overnight broth culture, which was prepared as mentioned in 2.3.1 was used. At the end of cultivation, the cell density was determined to be 1 x 10^8 cells/ml using the Jacobsen et al. (2011) method. A 2 ml sample of the culture broth was used to extract 3-hydroxy fatty acids. The extraction and analysis procedures were based on Madu et al. (2015) method. The 2 ml sample was transferred to a 15 ml centrifuge tube (Becton-Dickinson Labware, United States) where the pH was thereafter decreased to 4 using 3 % formic acid. A volume of 2 ml methanol-chloroform (HPLC-grade; Merck, United States) cocktail solution (1:1, v/v) was added to the centrifuge tube containing the culture. The suspension was vortex-mixed and allowed to stand for 20 min. This step was followed by the addition of 2 ml distilled water to the same centrifuge tube. The suspension was allowed to stand for a further 20 min. The suspension was then centrifuged (HERMLE Labortechnik, Germany) at 13000 x *g* for 15 min to fractionate. The chloroform layer (wherein the 3-hydroxy fatty acid are mobilised), was aspirated and dispensed into a sterile 15 ml centrifuge tube. Impurities were then removed by adding ± 2 spatulas of sodium sulphate in the sample, and thereafter the sample was moved to a clean test tube. The fraction was dried under a stream of nitrogen in a fume hood. Ten independent extractants were obtained from each tested isolate. The extractants were reconstructed in 1 ml of 95% methanol. The analytical 3-hydroxy fatty acid standards, i.e. 3-OH C8:0, C9:0, C10:0, C12:0, C14:0, and C16:0 (Larodan Fine Chemicals, Sweden) which were to be used for reference, were also reconstituted in 1 ml of 95% methanol.

High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) was performed by applying a 20 µl sample injection volume on to a Clupeus C18 (150 x 3 mm, 5 µm particle size) HPLC column (Higgins Analytical, United States) with a Shimadzu Prominence system coupled to a Sciex 4000QTRAP. Solvent A, HPLC grade water, and solvent B, HPLC grade methanol was acidified with 0.1% formic acid. The separation was performed using an 8 min 95% solvent B run. To analyse the samples, a targeted Multiple Reaction Monitoring (MRM) workflow was performed. The targeted analyses of all the 3-hydroxy fatty acids *viz*. were performed using the MRM transitions indicated in Table 1. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions are used as qualifiers. The qualifiers serve as an additional level of confirmation for the presence of the analyte; the retention time for these two transitions needs to be the same. All data acquisition and processing were performed using Analyst 1.5 (AB SCIEX) software.

To quantify the concentration extracted from each isolate, a value was extrapolated off a calibration curve. The curve was constructed using the values from each commercial standard through conducting a dilution series of the standards, followed by the extrapolation of the final concentrations from the calibration curve. The mean and standard deviation was calculated across the 10 independent replicates within each of the defined experimental classes. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p*-value less than 0.05 indicated abnormal distribution of the data points.

Table 1. Transitions used for targeted analysis of 3-hydroxy fatty acids by HPLC-MS.

3-Hydroxy fatty acid	Transition	
	Quantifier	Qualifier
3-hydroxyoctanoic acids (3-OH C8:0)	159.0 > 59.1	159.0 > 41.0
3-hydroxynonanoic acids (3-OH C9:0)	173.0 > 59.1	173.0 > 41.0
3-hydroxydecanoic acids (3-OH C10:0)	187.0 > 58.9	187.0 > 41.1
3-hydroxydodecanoic acids (3-OH C12:0)	215.1 > 58.9	215.1 > 41.1
3-hydroxytetradecanoic acids (3-OH C14:0)	243.1 > 59.1	243.1 > 41.1
3-hydroxyhexadecanoic acids (3-OH C16:0)	271.1 > 59.1	271.1 > 41.1

2.3.4 Statistical analysis

For each study, three independent experiments were performed, unless stated otherwise. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option. A *p*-value of less than or equal to 0.05 was considered significant. To this end, a value with a different alphabet to the other implies there is a significant difference while those with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p*-value less than 0.05 indicated abnormal distribution of the data points.

2.4 RESULTS AND DISCUSSION

2.4.1 The isolates revealed to be *Pseudomonas aeruginosa*

The amplicons, generated using the universal primer set (27f and 1472r), can be visualised in Fig 1. The amplicons had the expected band of 1500 bp. Upon sequencing and analysing the base sequences, the isolates were identified as *Pseudomonas aeruginosa* because they had 99% or 100% nucleotide similarities with *Pseudomonas aeruginosa* sequences deposited in the NCBI database.

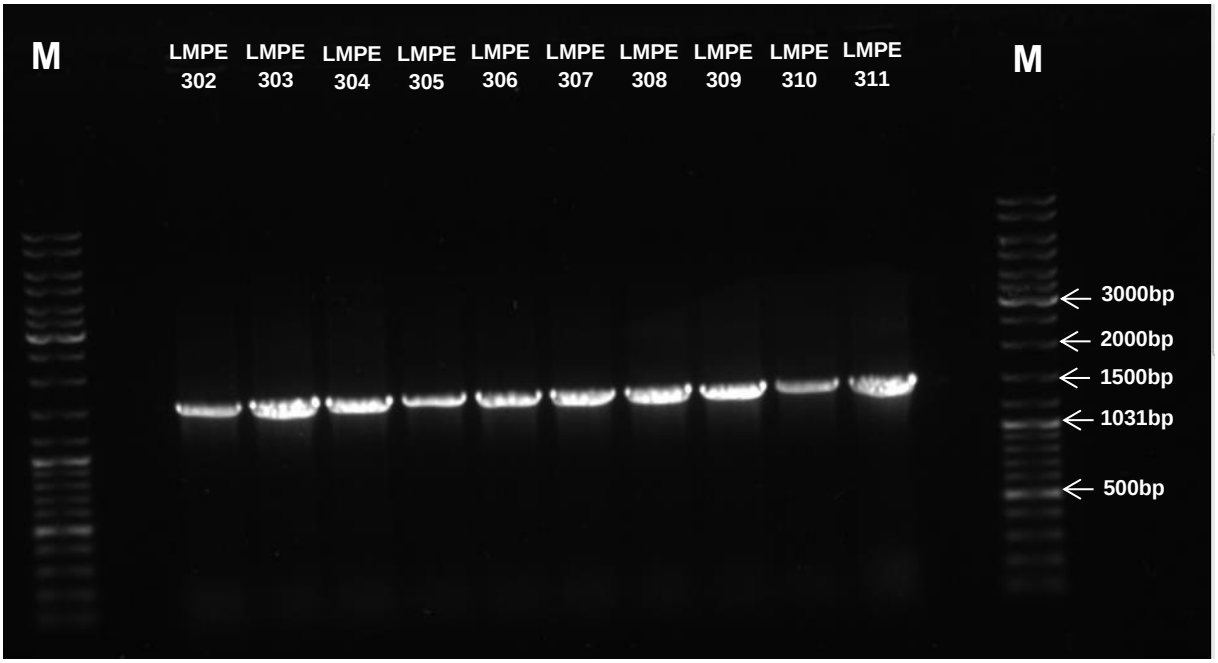

Fig 1. Electropherogram showing PCR products for the primer pairing 27f and 1472r. Lanes M = reference markers; lanes LMPE 302 - LMPE 311 = loaded DNA samples representing the clinical isolates; bp = base pairs.

The isolates were also shown to produce varying amounts of pyocyanin (Table 2), which is a unique phenazine pigment produced only by *Pseudomonas aeruginosa* (Baron and Rowe, 1981; Fourie *et al*., 2016). This water-soluble bluish-green virulence

factor (Dietrich *et al*., 2006) that has antimicrobial properties against numerous microbes (Baron and Rowe, 1981) and is highly toxic to eukaryotic cells (O'Malley *et al*., 2003). The tested isolates were observed to produce different concentrations of pyocyanin. Although the calculated numerical output for each clinical isolate, relative to the reference strain, suggested a great variation, the variation was not significant when the *p*-values were considered.

Table 2. Quantification of pyocyanin production by used *P. aeruginosa* isolates.

Strain description	**Pyocyanin (µg/ml per 10^8 cell density)**	**Amount produced by each strain relative to PA01**
PA01	2.53 (0.984)	100%
LMPE 302	1.02 (0.162)[a]	-60%
LMPE 303	3.47 (0.255)[a]	+37%
LMPE 304	2.20 (0.450)[a]	-13%
LMPE 305	3.79 (3.270)[a]	+50%
LMPE 306	1.59 (0.223)[a]	-37%
LMPE 307	0.86 (0.306)[a]	-66%
LMPE 308	3.82 (1.263)[a]	+51%
LMPE 309	2.13 (0.487)[a]	-16%
LMPE 310	3.86 (3.214)[a]	+53%
LMPE 311	3.32 (0.478)[a]	+31%

[a] = Not significantly ($p > 0.05$) different to PA01. Values in brackets are the standard deviations.

2.4.2 3-Hydroxy fatty acid extraction, analysis, and relative quantification

The initial results from the HPLC-MS analysis yielded extracted ion chromatographs (EIC) that show the elution profile and retention time, together with the mass spectrum that indicated the specific diagnostic peaks (deprotonated molecular ion [M-H]-, hydrogen-bound dimer ion ([M+M-H]-), and a sodium-bridged dimer ion ([M+Na+M-H]-)) for each of the specific commercial analytical standard compound, *i.e.* 3-hydroxy C8:0, 3-hydroxy C9:0, 3-hydroxy C10:0, 3-hydroxy C12:0, 3-hydroxy C14:0 and 3-hydroxy C16:0. The above information, summarised in Table 3, was used as a reference to identify the nature of the each extractant. It was observed that all 3-hydroxy fatty acids analysed were produced by the different isolates, except for 3-hydroxy tetradecanoic acid. Additionally, isolates LMPE 302 and LMPE 304 did not produce any 3-hydroxy nonanoic acid.

The extrapolated concentration that each isolate was able to produce is summarised in Table 4. It was observed that each of the isolates produced varying concentrations of the different 3-hydroxy fatty acid species. Only isolate LMPE 310 produced a concentration of a species that could be prepared in the laboratory for further use. The species was 3-hydroxydecanoic acid (3-OH C10:0) and the estimated concentration was 1 µM concentration. The concentration was obtained from a sample with a density of 1 x 10^8 cells/ml.

Table 3. Molecular annotation of analysed 3-hydroxy fatty acids.

Sample details			**Characterisation of 3-OH fatty acid**		
Nature of used standards	Replicates	RT (min)	Diagnostic MS/MS peaks		
			$[M-H]^-$	$[M+M-H]^-$	$[M+Na+M-H]^-$
(3-OH C8:0)	*n* = 10	3.21	159.088	319.246	342.236
(3-OH C9:0)	*n* = 10	3.31	173.232	347.472	370.462
(3-OH C10:0)	*n* = 10	3.47	187.259	375.526	398.519
(3-OH C12:0)	*n* = 10	3.93	215.309	431.626	454.616
(3-OH C14:0)	*n* = 10	4.70	243.367	487.742	510.732
(3-OH C16:0)	*n* = 10	5.94	271.415	543.838	566.828

3-OH = 3-hydroxy fatty acids; RT = retention time

Table 4. Tabulation of the different 3-hydroxy fatty acids species produced and respective concentrations.

Isolates	3-Hydroxy fatty acid concentration (μM)					
	3-OH C8:0	3-OH C9:0	3-OH C10:0	3-OH C12:0	3-OH C14:0	3-OH C16:0
PA01	0.086 (0.003)[a]	0.020 (0.002)[a]	0.187 (0.006)[a]	0.035 (0.004)[a]	-	0.021 (0.005)[a]
LMPE 302	0.013 (0.002)[b]	-	0.001 (0.001)[b]	0.001 (0.001)[b]	-	0.005 (0.005)[a]
LMPE 303	0.003 (0.003)[b]	0.013 (0.005)[a]	0.051 (0.120)[b]	0.014 (0.073)[a]	-	0.084 (0.001)[b]
LMPE 304	0.074 (0.028)[a]	-	0.167 (0.011)[a]	0.123 (0.007)[b]	-	0.053 (0.010)[a]
LMPE 305	0.102 (0.029)[a]	0.020 (0.005)[a]	0.327 (0.020)[b]	0.073 (0.009)[b]	-	0.058 (0.005)[b]
LMPE 306	0.354 (0.005)[b]	0.037 (0.002)[a]	0.566 (0.006)[b]	0.087 (0.003)[b]	-	0.031 (0.006)[a]
LMPE 307	0.054 (0.005)[a]	0.021 (0.007)[a]	0.574 (0.009)[b]	0.168 (0.007)[b]	-	0.050 (0.007)[a]
LMPE 308	0.020 (0.003)[b]	0.015 (0.005)[a]	0.088 (0.015)[a]	0.014 (0.009)[a]	-	0.057 (0.005)[b]
LMPE 309	0.062 (0.006)[a]	0.012 (0.006)[a]	0.181 (0.001)[a]	0.096 (0.009)[b]	-	0.050 (0.002)[a]
LMPE 310	0.123 (0.028)[b]	0.021 (0.007)[a]	1.026 (0.231)[b]	0.370 (0.007)[b]	-	0.049 (0.005)[a]
LMPE 311	0.012 (0.003)[b]	0.019 (0.006)[a]	0.574 (0.103)[b]	0.077 (0.005)[b]	-	0.034 (0.002)[a]

Cultures were standardised to 1 x 10^8 cell/ml before extraction. There was normality of data distribution per all the experimental classes. [a] = Not significantly ($p > 0.05$) different to PA01; [b] = Significantly ($p < 0.05$) different to PA01. Values in brackets are the standard deviations.

In conclusion, the current study allowed for the confirmation of the isolates' identity by detecting the presence of pyocyanin, a pigment that is associated with pseudomonal cells, as well as by analysing their nucleotide sequence and matching it to that of *P. aeruginosa*. The analyses of the lipid extractants revealed the presence of 3-hydroxy fatty acids, the nature of which was limited by the standard used. It is possible that other 3-hydroxy fatty acid species were present but, not detected. It was reasoned that the detected 3-hydroxy fatty acid species were secreted in free-form based on the extraction protocol. This is because Knirel et al. (2006) and Pier (2007) reported that 3-hydroxydodecanoic acid (3-OH C12:0) is the most abundant fatty acid in the lipid A fraction rather than 3-OH C10:0. It is possible that 3-OH C12:0 was not detected in large quantities, possibly because it was still attached to the LPS. This also supports the idea that the detected 3-OH C10:0 was not cleaved from the LPS. Despite the above, it is prudent to also design future studies that include a sample LPS as an additional experimental condition for comparison purposes. This is more so, as it was intended to determine if 3-OH C10:0 could invoke the pro-inflammatory response.

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CHAPTER 3

Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vitro* studies

Manuscript based on this chapter has been prepared and will be submitted to Frontiers in Immunology for consideration.

3.1 ABSTRACT

In the previous chapter, it was shown that pseudomonal cells could produce 3-hydroxy fatty acids. These oxygenated lipid-based molecules have been implicated in the pathogenesis of numerous microbes. Towards this end, the current study sought to investigate if pseudomonal 3-hydroxydecanoic acid (3-OH C10:0) may also promote the pathogenesis of this organism. The obtained data suggested that, similarly to the lipopolysaccharide (LPS), 3-OH C10:0 might function as a pro-inflammatory molecule after a murine macrophage cell line was exposed to this molecule. To be specific, 3-OH C10:0-treated macrophages induced significant phosphorylation levels of mitogen-activated protein kinase (MAPK) p38 ($p < 0.05$) and interferon-gamma (IFN-γ; $p < 0.05$) production, when compared to non-treated macrophages. The molecule, 3-OH C10:0, further suppressed fetuin A (FetA) *(*$p < 0.05$) levels produced by macrophages. This protein is essential for enhancing the internalisation of particles by phagocytes. Thus, it was not surprising that the pHrodo™ staining results revealed that fewer pseudomonal cells were internalised ($p < 0.05$) by macrophages in the presence of this fatty acid than in its absence. Interestingly, the pseudomonal cells that were internalised, seemed (in the presence of 3-OH C10:0) to survive the phagocytic event – as significantly more colonies ($p < 0.05$) were recovered, when compared to colonies obtained in the absence of 3-OH C10:0. The obtained results point towards a duality in function wherein the molecule can signal for inflammatory processes, and yet promote intracellular survival following internalisation.

Keywords: 3-hydroxydecanoic acid (3-OH C10:0), Macrophages, Phagocytosis, Pro-inflammation, *Pseudomonas aeruginosa*

3.2 INTRODUCTION

3-Hydroxy fatty acids are oxygenated lipids that are characterised by a hydroxyl group on the beta carbon (Kock *et al*., 2003, 2007; Sebolai *et al.,* 2007, 2012). These molecules may be (un)saturated, (un)branched or even linked to other macromolecules (Bhatt *et al*., 1998; Kock *et al*., 2003). The amphiphilic quality of these molecules is evidenced when immersed in aqueous environments (Kock and Botha, 1998). 3-Hydroxy fatty acids, in a free-form, have been reported to be widely distributed in fungi, thus; most of our current knowledge stems from studies conducted in fungi (Stodola *et al*., 1967; Vesonder *et al*., 1968; Kurtzman *et al*., 1974; Kock *et al*., 2000, 2007). In determining their function, these compounds were shown to be mainly involved in the sexual stages of many fungi (Kock *et al*., 1998, 2003). However, in the pathogenic *Cryptococcus neoformans*, 3-hydroxy fatty acids were shown to be associated with the capsule and played a role in impairing amoebal phagocytosis (Madu *et al*., 2015, 2017).

There is limited information regarding bacterial 3-hydroxy fatty acids, especially those that occur in a free-form, in the context of pathogenesis. In their paper, Kutschera suggested that following the production of 3-hydroxy fatty acids, these molecules may be trafficked extracellularly via the action of outer membrane vesicles (Kutschera *et al*., 2019). Upon release to the extracellular environment, they may act as signalling molecules (Flavier *et al*., 1997). In the context of *P. aeruginosa*, its cells can secrete hydroxy fatty acids that can promote its survival by appropriating environmental advantage over some bacterial species (Hou, 2008, Davies and Marques, 2009) or promote its pathogenesis (Govan and Deretic, 1996).

Against this background, the current study aimed to expand on the above, and determine the possible function(s) of pseudomonal 3-hydroxydecanoic acid (3-OH C10:0) on a commercial, murine macrophage cell line. Macrophages are professional, mononuclear immune cells that resolves foreign, infectious agents that might be present in a mammalian host (Shi and Pamer, 2014; Uribe-Querol and Rosales, 2017). To the point of the current study, it was sought to determine if 3-OH 10:0 may have a pro-inflammatory quality, which might promote further immunological development. For this part of the study, crude lipopolysaccharide (LPS) sample and LPS sugar component (α-D-glucosamine-1-phosphate) were included for comparison purposes. In the end, the effect of 3-OH C10:0 in assisting pseudomonal cells to survive a phagocytic event, was also assessed.

3.3 MATERIALS AND METHODS

3.3.1 Cultivation and standardisation of cells

The clinical isolate, *Pseudomonas aeruginosa* LMPE 310, was used in the study. The organism was streaked out on a nutrient agar (NA, 23 g/l, Sigma-Aldrich, United States) plate, and incubated at 37 ºC for 24 h. A loopful of the cells, from the 24 h old agar plate, was scooped and used to inoculate a 50 ml centrifuge tube (Becton-Dickinson Labware, United States) that contained 25 ml of nutrient broth (NB, 25 g/l, Sigma-Aldrich, United States). The inoculated tubes were incubated overnight at 37 ºC while shaking at 160 rpm on an orbital shaker. For standardisation of the cells, the optical density (OD) of each culture was first measured at OD_{600nm} using WTW PhotoLab S6 (LABTEC, United States) photometer. The obtained reading was substituted into the equation; cells/ml = OD_{600} x 2.5 x 10^8, by Jacobsen et al. (2011)

to determine the number of colony-forming units per millilitre (cells/ml). With the equation, the cell concentration of *Pseudomonas* cells was adjusted with phosphate-buffered saline (PBS; Oxoid, South Africa) to reach a final cell concentration of 1 x 10^6 cells/ml. This was the initial determined cell density prior to any further incubation or experimental work.

The RAW 264.7 macrophage cell line (originally obtained from ATCC) was cultivated in RPMI-1640 medium that was supplemented with 10% foetal bovine serum (Biochrom, Germany), 20 U/ml penicillin, 20 mg/ml streptomycin (Sigma-Aldrich, United States) and 2 mM L-glutamine (Sigma-Aldrich, United States). For each biological repeat, the cells were grown (37 °C and 5% CO_2) until they reached 80% confluence and their viability determined using the trypan blue stain. The macrophages were then standardised using a haemocytometer to reach a final cell concentration of 1 x 10^6 cells/ml in 10 ml of fresh RPMI-1640 media. A 100 µl suspension of macrophages was seeded into wells of a sterile, disposable 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany) and left overnight in a 5% CO_2 incubator at 37 °C. The next day, the media was aspirated, and 100 µl of fresh RPMI-1640 media that was spiked with 100 ng/ml of crude LPS sourced from *E. coli*, was added to the wells for the activation of the macrophages prior to the initiation of the experiment. The cells were allowed to interact with the *E. coli* LPS for 6 h in a 5% CO_2 incubator at 37 °C. At the end of 6 h, the spiked media was aspirated and 100 µl of fresh RPMI-1640 media was dispensed to the same wells.

3.3.2 Compounds

The 3-OH C10:0 standard, was purchased from Larodan Fine Chemicals (Sweden). The compound was first dissolved in ethanol (Saarchem, South Africa) and further diluted in LPS-free water (Sigma-Aldrich, United States). The compound was tested at final concentrations of 1 µM and 1000 µM in RPMI-1640. The final concentration of ethanol in RPMI-1640 media never exceeded 1%. The pseudomonal LPS was obtained as a crude sample from Sigma Aldrich, and it was reconstituted in LPS-free water to prepare a stock solution of 1 mg/ml. The LPS was tested at a final concentration of 1 µg/ml. An LPS sugar component (α-D-glucosamine-1-phosphate) was also obtained from Sigma-Aldrich. The α-D-glucosamine-1-phosphate was dissolved in LPS-free water to prepare a 1 mM stock solution, which was tested at a final concentration of 1 µM.

3.3.3 The effects of 3-OH C10:0 on a murine macrophage cell line

3.3.3.1 Metabolic activity

The stimulated macrophages (1 x 10^6 cells/ml seeded in 100 µl of media, inside the wells) were challenged with 100 µl of 3-OH C10:0. These immune cells were either exposed to 1 µM (amount secreted by 1 x 10^8 pseudomonal cells; as shown in Chapter 2) or 1000 µM (used as an upper limit concentration). In separate experiments, macrophages were also exposed to 1 µM of α-D-glucosamine-1-phosphate or 1 µg/ml of the pseudomonal LPS. The microtiter plate was then incubated for 6 h at 37 ºC in a 5% CO_2 incubator. The treated macrophages were reacted with 50 µl of the tetrazolium salt (XTT; Sigma-Aldrich, United States), in the presence of 4 µl menadione (Sigma-Aldrich, United States), to analyse their metabolic activity. After 3

h of initiating the tetrazolium reaction (37 ºC in a 5% CO_2 incubator), the absorbance readings were measured at 492 nm using a spectrophotometer. Non-treated macrophages were also included for reference in determining the shift in the metabolic activity.

3.3.3.2 Production of pro-inflammatory markers

The stimulated macrophages (1 x 10^6 cells/ml seeded in 100 µl of media, inside the wells) were treated in a similar manner as mentioned section 3.3.3.1. After 6 h of incubation (37 ºC in 5% CO_2), the supernatant was gently aspirated (to avoid disrupting the settled monolayer) and transferred to wells on an ELISA plate that was coated with antibodies specific for interferon-gamma (IFN-γ; Sigma-Aldrich, United States). For the mitogen-activated protein kinase (MAPK) p38 assay, the remaining monolayer was lysed using 100 µl of 1x complete lysis buffer that was supplied with the MAPK p38 ELISA kit (Sigma Aldrich, United States). The lysate was then transferred to wells that were coated with antibodies specific for MAPK p38. Each ELISA assay was performed according to the respective manufacturer's protocol. The absorbance of each plate was read at 450 nm. Non-treated macrophages were also included for reference in determining the shift in the levels of IFN-γ and phosphorylation levels of MAPK p38.

3.3.3.3 Production of fetuin A (FetA)

The stimulated macrophages (1 x 10^6 cells/ml seeded in 100 µl of media, inside the wells) were treated in a similar manner as mentioned section 3.3.3.1. After 6 h of incubation (37 ºC in 5% CO_2), the supernatant was gently aspirated (to avoid disrupting the settled monolayer) and transferred to wells on an ELISA plate that was

coated with antibodies specific for FetA (Sigma-Aldrich, United States). The ELISA assay was performed according to the manufacturer's protocol. The absorbance of the plate was read at 450 nm. Non-treated macrophages were also included for reference in determining the shift in the levels of FetA.

3.3.3.4 Internalisation efficiency assay: an indicator of pseudomonal engulfment

The ability of macrophages to internalise pseudomonal cells (strain LMPE 310) was analysed using pHrodo™ Green Zymosan A BioParticles (Life Technologies, United States). In brief, the standardised pseudomonal cells (1 x 10^6 cells/ml) in 999 µl of RPMI 1640 medium were stained with 1 µl of pHrodo™ Green stain for 1 h. These cells, in 1.5 ml plastic tubes, were incubated at 37 ºC while slowly being agitated on an orbital shaker. After the 1 h incubation period, the pseudomonal cells were washed with PBS twice and re-suspended in 1000 µl of fresh RPMI-1640 medium. This fresh media contained either 2 µM or 2000 µM of 3-OH C10:0, 2 µg/ml of LPS or 2 µM of α-D-glucosamine-1-phosphate. A 100 µl suspension of the pseudomonal cells was aspirated and then dispensed into the wells that contained seeded macrophages (100 µl; 1 x 10^5 cells/ml). Before co-cultivation, stimulated macrophages were allowed to settle in the wells for 2 h at 37 ºC in a 5% CO_2. The plate, with co-cultured cells, was then incubated for 6 h at 37 ºC in 5% CO_2. At the end of the co-incubation, the non-internalised cells were removed by washing the wells twice, with PBS. The results were obtained by measuring the induced fluorescence (492 nm; ex/538 nm; em) using a Fluoroskan Ascent FL microplate reader. Non-treated macrophages were also included for reference in determining the shift in the relative fluorescence units (RFUs) readings.

3.3.3.5 Phagocytosis efficiency assay: an indicator of pseudomonal survival

The standardised pseudomonal cells (1 x 10^6 cells/ml) were suspended in RPMI-1640 media containing either 2 µM or 2000 µM of 3-OH C10:0, 2 µg/ml of LPS or 2 µM of α-D-glucosamine-1-phosphate. A 100 µl suspension of the pseudomonal cells was aspirated and then dispensed into the wells that contained the stimulated macrophages (100 µl; 1 x 10^5 cells/ml). The plate, with co-cultured cells, was then incubated for 6 h at 37 ºC in a 5% CO_2. At the end of the co-incubation, the non-internalised cells were removed by washing the wells twice, with PBS. Macrophages were allowed to stand in triton 100-X for 10 min in order to lyse them. After which, the lysate was aspirated and transferred to 1.5 ml plastic tubes, and 1 in 10 dilutions were made. A volume of 100 µl of the dilution was spread out onto an agar plate, incubated at 37 °C for 24 h and colony-forming units were counted. Colonies of pseudomonal cells, from co-cultures prepared in the absence of the different compounds, were counted in order to deduce the shift in the survival of pseudomonal cells.

3.3.4 Statistical analysis

For each study, three independent experiments were performed, unless stated otherwise. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option. A *p*-value of less than or equal to 0.05 was considered significant. To this end, a value with a different alphabet to the other implies there is a significant difference while those with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p*-value less than 0.05 indicated abnormal distribution of the data points.

3.4 RESULTS AND DISCUSSION

3.4.1 3-OH C10:0 is not detrimental to RAW 264.7 macrophages

The response of macrophages to the specific test compounds is summarised in Fig. 1. The results show that 3-OH C10:0 at 1 µM, did not significantly (p = 0.134) reduce the metabolic activity of macrophages when compared to the non-treated macrophages. However, an increase in the 3-OH C10:0 concentration to 1000 µM, led to a more than 50% reduction (p = 0.002) in the metabolic activity of macrophages. A future study that measures the release of the cytosolic enzyme, lactate dehydrogenase, into the cultivation media, should be included. Results obtained from such a study ought to be considered together with the XTT results in order to infer on the toxicity of this fatty acid.

Fatty acids are important membrane components as well as sources of energy (Wakil and Abu-Elheiga, 2009). Therefore, exposure of cells to exogenous fatty acids may impact regulatory mechanisms that govern fatty acid metabolism. To illustrate this, diets rich in fatty acids may reduce expression of lipogenic enzymes (Schoonjans *et al*., 1995), and macrophages treated with fatty acids have been reported to show changes in metabolism (Curi *et al*., 1993; Bond *et al*., 1995). In the context of the current study, the data suggests that 3-OH C10:0, at concentrations estimated to be secreted by pseudomonal cells, would not be detrimental to the metabolic activity of macrophages, and possibly the consistency of the membrane fluidity.

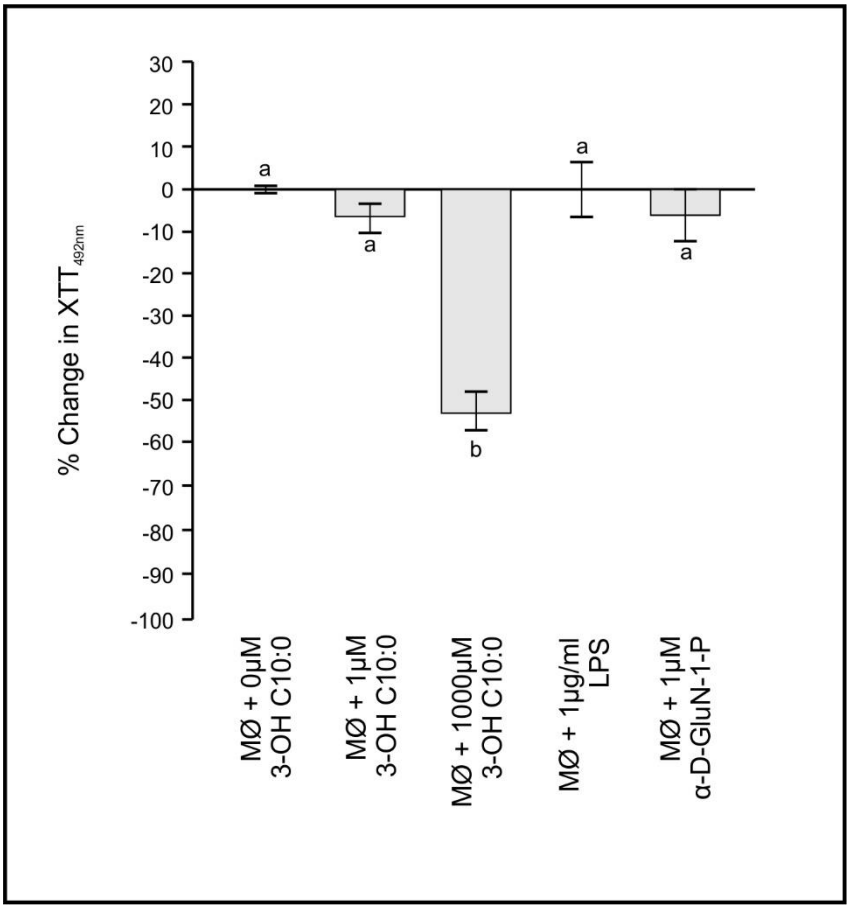

Fig 1. The effect of 3-OH C10:0 on macrophages. The measured XTT absorbance readings were used as an indicator of macrophage metabolic activity. MØ = macrophage. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b] = significantly different ($p < 0.05$).

3.4.2 3-OH C10:0 immunologically sensitises the RAW 264.7 macrophages

The MAPK p38 results are shown in Fig. 2a. When considering the results of the control (non-treated macrophages), the data were comparable to that of the α-D-glucosamine-1-phosphate, since both were not statistically significant ($p = 0.667$). This implied that α-D-glucosamine-1-phosphate may be immunologically silent. On the other hand, the LPS was able to lead to increased phosphorylation levels ($p = 0.017$)

of this inflammatory marker, when compared to non-treated macrophages. Increased phosphorylation levels, were also observed after the macrophages were exposed to 1 µM (p = 0.047) and 1000 µM (p = 0.009) 3-OH C10:0. Given the strong link that exists between MAPK p38 pathway and inflammation (Yang *et al*., 2014), it was theorised that an increase in the activity of this protein (as a result of exposure to 3-OH C10:0, including the LPS) would, in turn, lead to elevated levels of pro-inflammatory cytokines. To test the idea, IFN-γ, which is a crucial cytokine that assists in pathogen clearance (Kak *et al*., 2018) was, subsequently, targeted in the study. When considering the IFN-γ data (Fig. 2b), it was apparent that all test compounds were able to significantly increase the levels of IFN-γ, although to different degrees based on the obtained numerical output for each compound. To this end, when compared to the non-treated macrophages, the α-D-glucosamine-1-phosphate (p = 0.017) increased the IFN-γ levels by 10%, 3-OH C10:0 at 1 µM by 63% (p = 0.005) and at 1000 µM, the levels were by increased 75% (p = 0.005). The LPS gave the highest numerical output that translated into an increase of 88% (p = 0.003). Based on Fig. 2, it is reasonable to conclude that the profile of 3-OH C10:0 mirrored that of the LPS, suggesting that this fatty acid may have a pro-inflammatory quality, and may also be the immunoregulatory centre of the LPS, as suggested by Kutschera et al. (2019). This finding is consistent with literature, wherein pseudomonal lipid A is reported critical for the triggering of immunity and the removal of the ester-linked acyl chains (3-hydroxy fatty acids) from the lipid A abolishes this activity (Ranf *et al*., 2015; Kutschera *et al*., 2019).

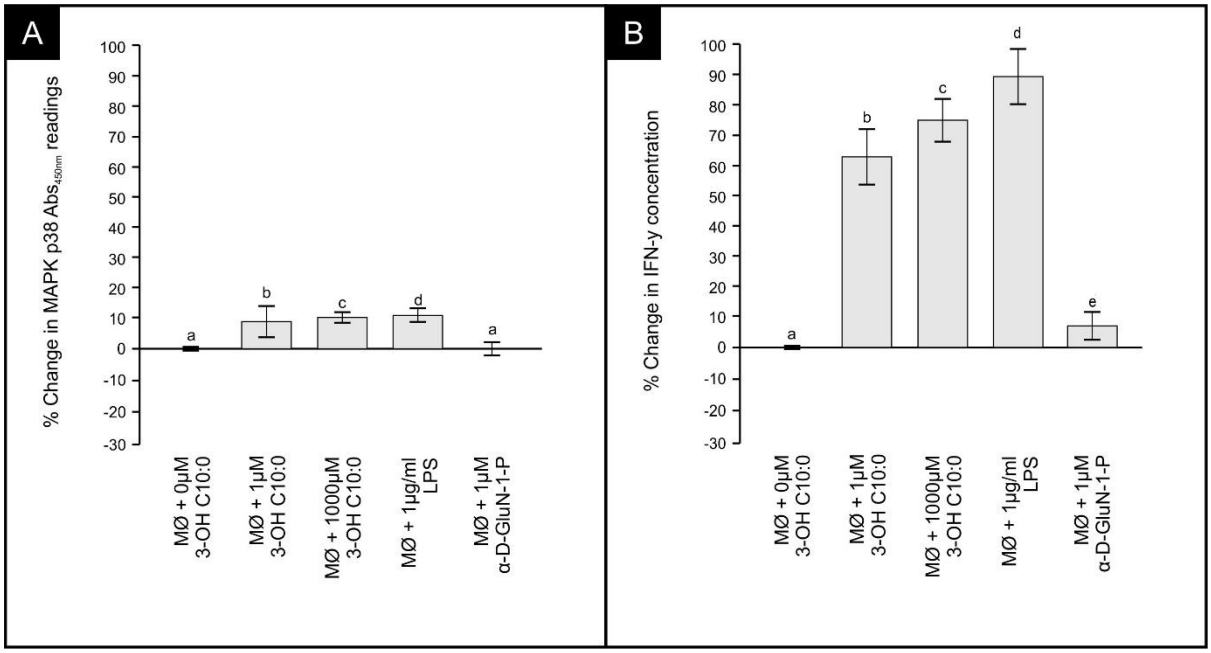

Fig 2. The effect of 3-OH C10:0 on pro-inflammatory mediators. (**A**) Indicates the levels of phosphorylated MAPK p38 while (**B**) illustrates the production of IFN-γ. MØ = macrophage. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b, c, d, e] = Significantly different ($p < 0.05$).

3.4.3 3-OH C10:0 impairs the RAW 264.7 macrophage phagocytosis

The ability of RAW 264.7 macrophages to produce FetA, a molecule that is reported to be essential for enhancing the internalisation of particles (Wang *et al*., 1998), was first assessed (Fig. 3). When considered against the non-treated macrophages, the analysed 3-OH C10:0 data suggested that there was impairment of cell uptake. To the point, the levels of FetA were significantly suppressed in the presence of 1 µM by 12% ($p = 0.041$) and 1000 µM by 17% ($p = 0.015$). The observation of fatty acids impeding phagocytosis has been noted elsewhere. In one study, short chained fatty acids were reported to prevent the release of lysozymes (Eftimiadi *et al*., 1987), and in another study, fatty acids reduced hydrogen peroxide production thus impairing phagocytosis

(Bellinati-Pires *et al*., 1993). The LPS and α-D-glucosamine-1-phosphate data were determined to be statistically insignificant ($p > 0.05$), the data implied that the two molecules would not interfere with the uptake process of pseudomonal cells.

To confirm the FetA results, pHrodo™ Green Zymosan A BioParticles was performed, and the results are summarised in Fig 4. The stain allows for cells that are captured and then internalised by phagocytic cells to fluoresce inside the acidic environment of the phagosome. Therefore, the read relative fluorescence units were used to estimate the efficiency of macrophages to internalise cells. As expected, the lower levels of FetA, in the presence of 3-OH C10:0, were associated with less pseudomonal cells being internalised. To the point, when compared to non-treated macrophages, 1 µM 3-OH C10:0 led to an 11% ($p = 0.011$) decrease in the internalisation efficiency while at 1000 µM the decrease was 19% ($p = 0.003$). Both the LPS and α-D-glucosamine-1-phosphate data was observed to be statistically insignificant ($p > 0.05$), this goes together with what was observed with the FetA results, these two molecules seem not to affect macrophage internalisation. The cells that did not fluoresce (as seen in Fig. 4) were assumed to have not been internalised and were eliminated by the washing step in the preparation of experiment, or if they were internalised, they were killed by the macrophages or may have escaped.

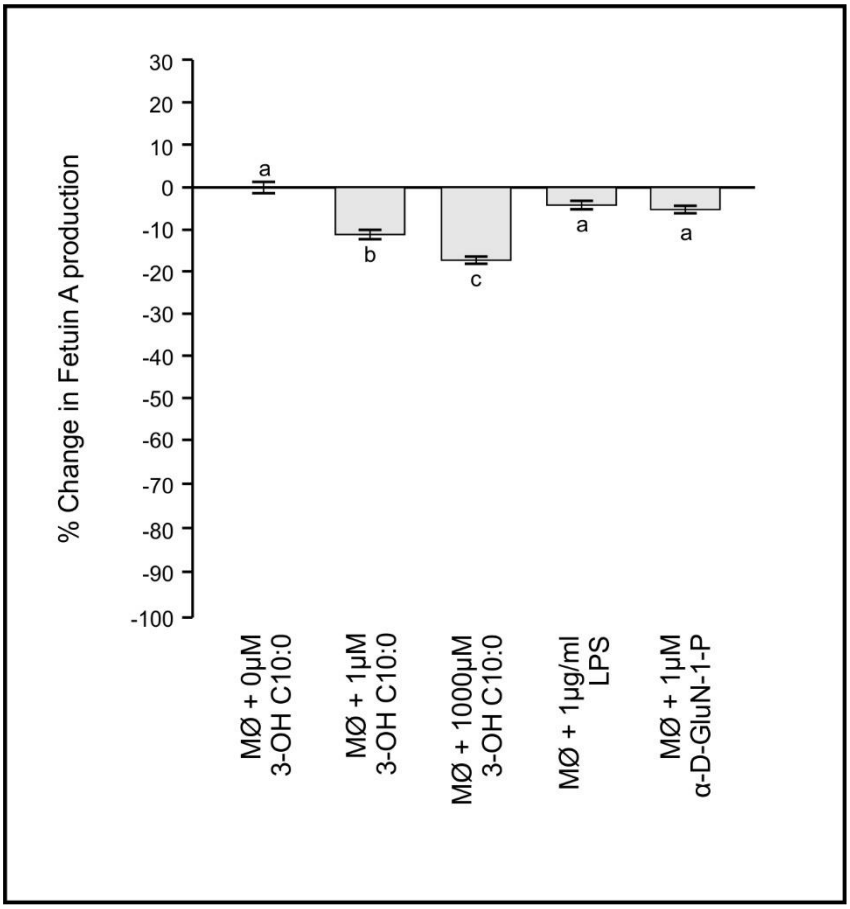

Fig 3. The effect of 3-OH C10:0 on FetA production. The reading that was given by non-treated macrophages was considered as background, and thus subtracted for normalisation purposes. MØ = macrophage. 3-OH C10:0 = 3-hydroxdecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b, c] = Significantly different ($p < 0.05$).

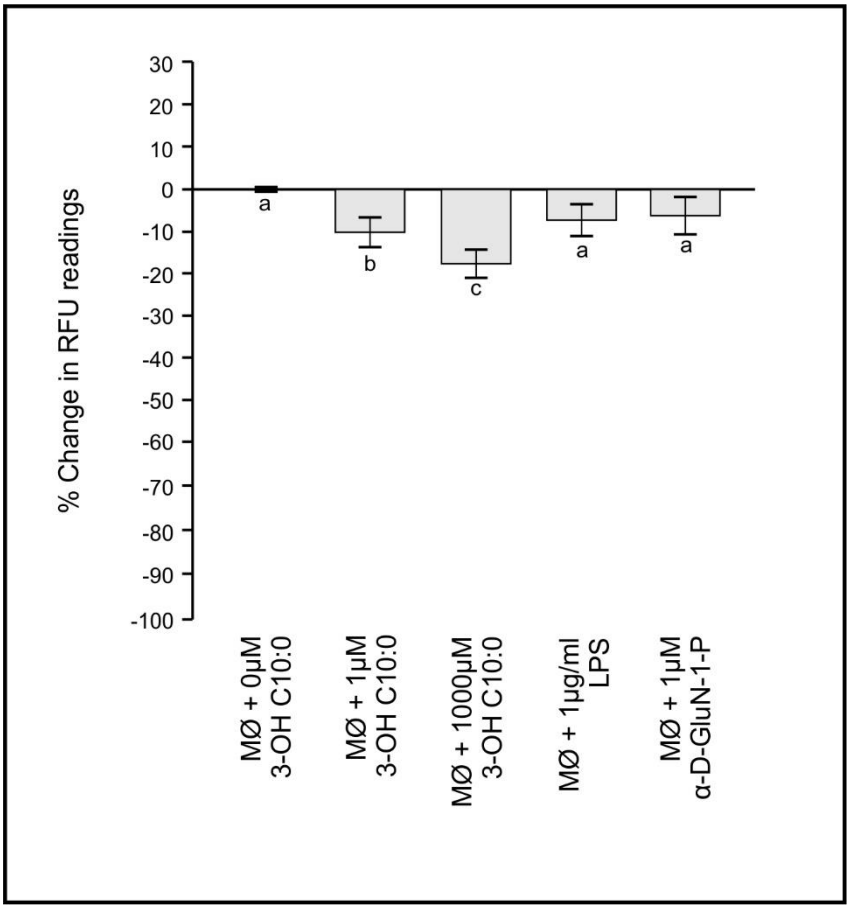

Fig 4. The effect of 3-OH C10:0 on macrophage internalisation efficiency. The test used the pHrodo™ stain, and the obtained relative fluorescence units were considered an indicator of efficiency. The reading that was given by non-treated macrophages was considered as background, and thus subtracted for normalisation purposes. MØ = macrophage. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b, c] = Significantly different ($p < 0.05$).

Therefore, to understand the fate of pseudomonal cells following co-cultivation with macrophages, a survival assay was performed by harvesting the internalised pseudomonal cells from the macrophages, plating out on agar plates and counting recovered colonies. The findings for this experiment are presented in Fig 5. The increasing concentrations of 3-OH C10:0 led to increased recovery ($p < 0.05$) of pseudomonal colonies on agar plates. When Fig. 5 data is considered with Fig. 4, it is reasonable to conclude that the high number of recovered colonies suggests that the cells were not killed by the macrophages but rather survived the phagocytic effect by escaping the phagosome or the 3-OH C10:0 may have shielded the cells from the harsh internal environment of the phagosome. Previously, our group has shown that 3-OH fatty acids could shield cells against hydrogen peroxide and hydrolytic enzymes (Madu *et al*., 2017). It is now important to also assess how 3-OH C10:0 affect other key processes in the maturation of phagosomes.

In conclusion, microbes have evolved numerous ways to subvert the action of the immune system; these mechanisms can be highly specific to each microbe (Sansonetti and Di Santo, 2007; Woodlard and Frelinger, 2008). It has been reported that microbes can deploy different mechanisms (alteration of microbial surfaces and secretion of microbial factors) that may function in concert to circumvent the action of immune cells – and this advantage provides the ideal situation for the manifestation of a successful infection (Cirl *et al*., 2008; Carlin *et al*., 2009; Bateman and Seed, 2010; Pollak *et al*., 2012; Shi and Pamer, 2014; Thakur *et al*., 2019).

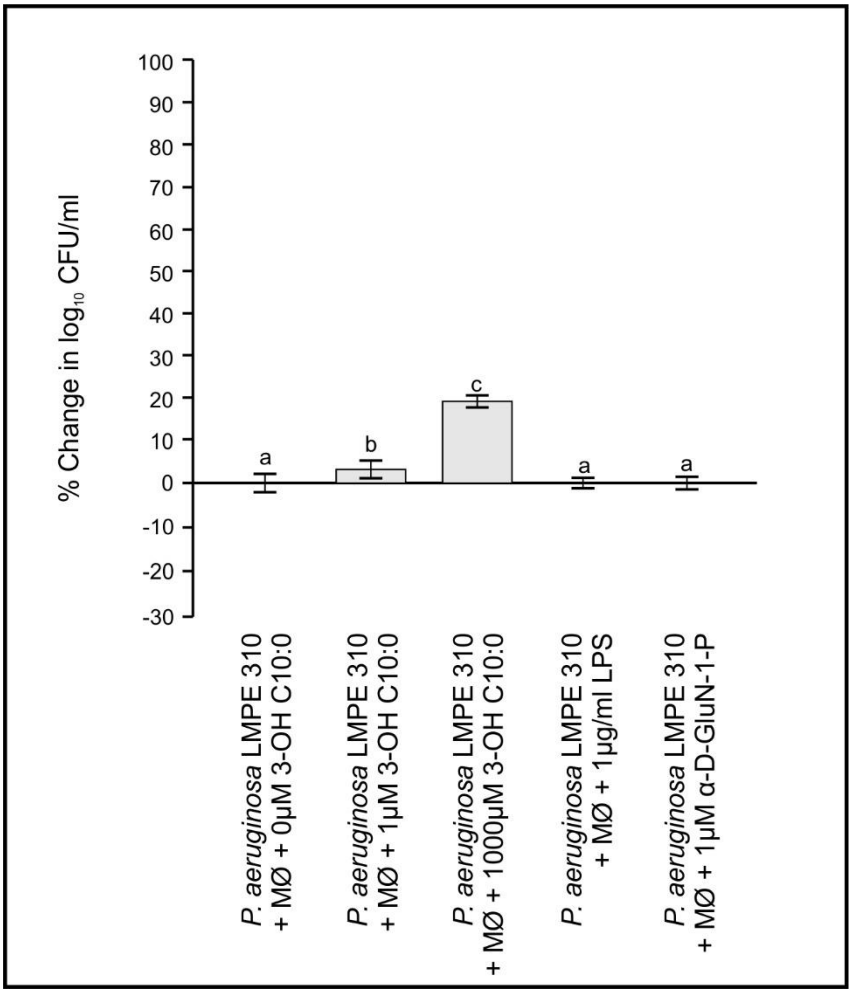

Fig 5. The effect of 3-OH C10:0 on the survival of pseudomonal cells. The counting of the recovered pseudomonal colonies was considered an indicator of pseudomonal cells surviving macrophage phagocytosis. MØ = macrophage. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b, c] = Significantly different ($p < 0.05$).

Concerning *Pseudomonas*, Kutschera et al. (2019) reported that the organism may use a secretory system to transport 3-hydroxy fatty acids to the extracellular environment – where they may affect infectious processes. This mechanism may well be demonstrated in the findings documented herein. It was demonstrated that 3-OH C10:0 is able to manipulate the MAPK p38 pathway, which controls cellular responses related to stress (Qi and Elion, 2005; Wang *et al*., 2012). In particular, the pathway is relevant to inflammatory diseases. Thus, it was not surprising to note that when macrophages were challenged with 3-OH C10:0, they significantly produced the pro-inflammatory IFN-γ to possibly promote anti-bacterial immunity. The 3-OH C10:0 seemed to stimulate the MAPK p38 pathway and IFN-γ production in a similar manner to the LPS. This raises curiosity regarding the importance of the 3-OH moiety to the LPS, since the immuno-modulatory centre of the LPS is located within the Lipid A, which has 3-hydroxy fatty acids. Garai et al. (2019) argued that while *Pseudomonas aeruginosa* is considered an extracellular pathogen solely, there is increasing evidence that shows its interaction with mammalian cells, including macrophages in the intracellular environment. In the context of macrophages, two bacterial factors, MgtC and OprF, have been identified that assist bacterial cells to escape from the phagosome (Garai *et al*., 2019). The MgtC protein promotes *P. aeruginosa* intracellular bacterial multiplication similar to *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) where it alters the bacterium's ability to translocate protons and to couple translocation to ATP synthesis (Belon *et al*., 2015). While the OprF, which is an outer membrane protein that provides protection against complement and lysozyme activity, which enables *P. aeruginosa* to evade killing and enhance cell invasion (Mittal *et al*., 2016). It is also conceivable that 3-hydroxy fatty acids may be working in concert with these factors to promote intracellular survival.

This is based on the observation that pseudomonal cells were able to survive better a phagocytic encounter in the presence of 3-OH C10:0. The possible interplay between 3-hydroxy fatty acids as well as MgtC and OprF should now be investigated. The action of 3-hydroxy fatty acids seems to be consistent when applied to amoeba and macrophages. In the amoeba study, these molecules were shown to neutralise the anti-microbial oxidative radicals (Madu *et al*., 2017). It would be interesting to determine how a whole organism may respond to the presence of 3-hydroxy fatty acids.

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CHAPTER 4

Elucidating the role of 3-hydroxy fatty acids (3-OH C10:0) in the pathogenicity of *Pseudomonas aeruginosa*: *In vivo* studies

Manuscript base on this chapter has been prepared and will be submitted to Frontiers in Microbiology for consideration

4.1 ABSTRACT

The use of animal models has been a crucial part of biomedical research providing insight into microbial pathogenesis as well as on the effect of microbial factors. In the current study, the direct effects of a 3-hydroxy fatty acid, 3-hydroxydecanoic acid (3-OH C10:0), was investigated on laboratory nematodes (immunocompetent and immunocompromised mutants) and rats. The 3-OH C10:0 was observed to be sufficiently potent to affect the survival of all nematodes when compared to the non-treated nematodes. The non-treated nematodes achieved a 70% survival rate at the time of terminating the study. The 3-OH C10:0, at 1000 µM, was shown to engage a signalling programme that involved the participation of mitogen-activated protein kinase (MAPK) p38 more than the extracellular signal-regulated kinases (ERK) 1 and 2. This suggested that the molecule may have a pro-inflammatory quality. This observation was further supported by the results obtained in laboratory rats. Here, the molecule was shown to increase the number of circulating monocytes ($p < 0.05$) within 6 h as well as increase the production of interferon-gamma (IFN-γ) ($p < 0.05$) when compared to the levels produced by animals challenged with the drug vehicle control. Taken together, these results suggest that 3-OH C10:0 may possess properties that may be useful in studying the response of the immune system to pseudomonal-derived 3-hydroxy fatty acids.

Key words: 3-OH C10:0, ERK 1/2, IFN-γ, Inflammation, MAPK p38, Monocytes, Nematodes, Rats.

4.2 INTRODUCTION

Part of our understanding around the development of infectious diseases, including the role of microbial factors in eliciting infectious processes, emerges from studies that included an animal model (Swearengen, 2018). Importantly, in such studies, a model is used to mirror a specific disease aspect. Thus, the obtained results assist in furthering current knowledge (Swearengen, 2018).

Researchers often use vertebrates and invertebrates as experimental models when studying certain disease pathologies that are elicited by microbes, including their molecules. (Gonzalez-Moragas *et al*., 2015). One commonly used invertebrate model is the nematodes (Marsh and May, 2012); this is due to these organisms being relatively cheap to purchase and easy to maintain as culture in a laboratory setting. Moreover, they save time through their rapid reproduction and they are easy to handle during experiments (Corsi *et al*., 2015). Also counting in their favour is that, their genome has been fully sequenced, which provides genetic and biochemical information (Corsi *et al*., 2015). The close examination of the genetic and biochemical information also revealed the existence of some important analogous similarities between the innate immune system of nematodes and that of humans (Ermolaevaa and Schumachera, 2014).

Despite the above, nematodes still lack an adaptive immune response, as observed in humans (Kaletta and Hengartner, 2006). Therefore, information related to how secondary immunological development and immunological memory are built may be lost (Kaletta and Hengartner, 2006; Ermolaevaa and Schumachera, 2014; Perlman,

2016). To this end, the inclusion of a complex animal model may be more useful. There are numerous studies that opt to use small mammalian animals such as rats or mice since they possess an immune response that is mechanised in a similar manner to that of humans (Buras *et al*., 2005). It is also possible to include both models in the same studies, depending on the specific disease aspect being addressed.

In the previous chapter, the role of 3-hydroxy fatty acids in influencing the health and functioning of macrophages was investigated. In the current chapter, an attempt was made to expand on the findings presented in Chapter 3, and thus, laboratory nematodes and rats were directly challenged with 3-hydroxydecanoic acid (3-OH C10:0). The presence of this molecule in these organisms may elicit infectious processes that may modulate the immune response or kill a susceptible host.

4.3 MATERIALS AND METHODS

4.3.1 Compounds

The 3-OH C10:0 standard was purchased from Larodan Fine Chemicals (Stockholm, Sweden). The compound was first dissolved in absolute ethanol (Protea chemicals, South Africa) and further diluted in LPS-free water (Sigma-Aldrich, United States). The final concentration of ethanol in LPS-free water never exceeded 1%. The compound was tested at final concentrations of 1 µM (a concentration estimated to be produced by *P. aeruginosa* LMPE 310) and 1000 µM (used as an upper limit control). A pseudomonal lipopolysaccharide (LPS) and the LPS sugar component; α-D-glucosamine-1-phosphate, were purchased from Sigma Aldrich. The two compounds

were reconstituted in LPS-free water. The LPS was tested at a final concentration of 1 µg/ml while α-D-glucosamine-1-phosphate at 1 µM.

4.3.2 Models

4.3.2.1 Caenorhabditis elegans (nematode)

Two hermaphrodite strains were first obtained from the Caenorhabditis Genetic Center at the University of Minnesota, United States, and were kindly donated by Prof C. H. Pohl of the University of the Free State, South Africa. The first strain has a single deletion Δ(*glp-4*), which results in the abnormal germline proliferation (TeKippe and Aballay, 2010). Due to the early stage termination of germline proliferation, there is no production of sperm cells in these mutants (Krijgsveld *et al*., 2003). This temperature-sensitive allele (*glp-4*) results in wild-type worms when grown at 15 °C and germline devoid worms when grown a 25 °C (Krijgsveld *et al*., 2003). The second strain had a double deletion Δ(*glp-4*) Δ(*sek-1*). The deletion of the *sek-1* gene results in an immunocompromised mutant due to the disruption of the conserved mitogen-activated protein (MAP) kinase involved in the innate immune response to pathogens (Kim *et al*., 2002). The *sek-1* gene encodes a MAP kinase kinase that functions directly upstream of the *C. elegans* homolog of the mammalian p38 MAP kinase (Breger *et al*., 2007). Thus, the response of the double deletion mutant was compared to that of the single deletion mutant.

4.3.2.2 Rattus norvegicus (Wistar rats)

Twenty Wistar rats, *i.e.* ten females (200 g – 300 g) and ten males (300 g – 400 g), were used in the study. The animals were obtained from the animal facility at the University of the Free State, South Africa. Ethical approval was obtained to use the animals in the current study (Application no. UFS-AED2017/0077). The animals were housed in a controlled environment (21±1°C; humidity 60%; lights on from 08:00 until 20:00; food and water were made available *ad libitum*).

4.3.3 The effects of 3-OH C10:0 on laboratory nematodes

4.3.3.1 Survival assay

For the propagation of the nematodes, a lawn of *Escherichia coli* OP50 was created on the surface of nematode growth medium with agar (NGM; 2.5 g/l peptone, 3 g/l sodium chloride, 17 g/l agar; Sigma Aldrich, United States) (Wood, 1988). Each mutant was separately transferred to an agar plate and incubated at 15 °C for 7 days. At the end of the incubation period, M9 buffer (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 5 g/l NaCl, 0.25 g/l $MgSO_4.7H_2O$; Sigma Aldrich, United States) was used to wash off the nematode from the *E. coli* OP50 containing plates. The washed off nematodes were collected into a 15 ml centrifuge tube and were washed four times with the M9 buffer. The washing was done by gently shaking the tube for a few seconds and allowing the tube to stand for 10 min before the buffer was aspirated. Using a stereo microscope, sixty nematodes were counted and transferred into a single well of a six-well tissue culture plate. The wells contained 2 ml of a liquid medium, 80% M9 buffer and 20% brain-heart infusion (BHI; 7.8 g/l brain extract, 9.7 g/l heart extract, 2.5 g/l disodium phosphate, 2.0 g/l dextrose). The media, in the different wells, was already

supplemented with the different test compounds (3-OH C10:0, pseudomonas LPS and α-D-glucosamine-1-phosphate) at the specified final concentrations. The plates with the nematodes were incubated at 15 °C. The nematodes were monitored daily for 11 days and were scored as either alive or dead. To the point, nematodes were considered dead if they did not display movement in response to picking mechanical stimulation. Such a nematode was removed from the liquid medium.

4.3.3.2 Nematode physiological response

The nematodes were prepared as mentioned above and were subsequently transferred to wells of a six-well tissue culture plate that already contained media supplemented with test compounds at specified final concentrations. The nematodes were left to interact with compounds for a period of 6 h or 24 h at 15 °C. At the end of the incubation period, the nematodes were transferred to 2 ml plastic tubes and centrifuged at 2000 x *g* for 5 min. Depending on the analysis to be undertaken, a lysis buffer that was supplied inside either the extracellular signal regulated kinase 1 and 2 (ERK 1/2; Sigma-Aldrich, United States) or mitogen-activated protein kinase p38 (MAPK p38; Sigma-Aldrich, United States) ELISA kit was used to lyse the nematodes. In brief, the pellet was re-suspended in 500 µl of 1x complete lysis buffer that was supplemented with a protease inhibitor cocktail (Sigma Aldrich, United States). The plastic tube, containing the re-suspended pellet and lysis buffer, was incubated at 4°C for 30 min. Thereafter, the contents were next homogenised with 0.5 mm diameter zirconium glass beads (White Scientific, United States) on a microtube homogeniser (BeadBug, Benchmark Scientific Inc.) to achieve mechanical rupture. The tubes were centrifuged at 2000 x *g* for 5 min. The supernatant was aspirated and dispensed to wells that were coated with antibodies specific for either ERK 1/2 or MAPK p38 on an

ELISA plate. Each ELISA assay was performed according to its manufacturer's protocol. The absorbance of each plate was read at 450 nm. Non-treated nematodes were also included for reference to determine the shift in the levels of ERK 1/2 and MAPK p38.

4.3.4 The effects of 3-OH C10:0 on laboratory rats

4.3.4.1 Animal infection

The animals were divided into five groups, each composed of two females and two males. All animals were given a single dose of 1 ml of the test compound per 100 g of the body weight. Group I rats were the control group, which received 3-OH C10:0 drug vehicle, which was LPS-free water. Group II rats were challenged with 3-OH C10:0 at a concentration of 1 µM. Group III rats were challenged with 3-OH C10:0 at a concentration of 1000 µM. Group IV rats were challenged with LPS at a concentration of 1 µg/ml. Group V rats were challenged with α-D-glucosamine-1-phosphate, at a concentration of 1 µM. The above were delivered intravenous via the tail vein. All animals were allowed to interact with the injected compounds for 24 h with the blood samples withdrawn after 6 h and 24 h.

The weight of the animals was measured at the start and termination of the study. In addition, prior to blood sampling, the animals were visually inspected for signs of distress (loss of coat/hair). At the time of sampling (after 6 h and 24 h), each animal was deeply anesthetised in a drop jar using a liquid inhalant anaesthetic (Davis, 2008) on cotton/gauze with a mesh grid over it before drawing blood. The blood sample was dispensed into two collection tubes (contained the anticoagulant agent ethylenediaminetetraacetic acid (EDTA)). At the end of the study, the animals were

immediately euthanized using CO_2. All the blood samples were analysed within 2 h of collection.

4.3.4.2 Haematological analysis

A tube with a 300 µl blood sample (contained in the anticoagulant agent tube) was analysed on an ADVIA 2120i Haematology System (Siemens, Germany). The machine is a bench-top, flow cytometric analyser that was calibrated to measure monocytes. These cells pass through a laser flow cell and two-angle light scatter that is used to determine cell size and nuclear density that are detected and recorded on the basophil cytogram in which the x-axis reflects the nuclear complexity and the y-axis reflects cell size (Harris *et al*., 2005).

The last anticoagulant tube, containing a 300 µl blood sample, was centrifuged at 2000 x *g* for 15 min to separate the blood plasma. This collected plasma was subsequently used to perform an ELISA assay for the detection of interferon-gamma (IFN-γ). A volume of 50 µl of the plasma was aspirated and dispensed into wells that were coated with antibodies specific for IFN-γ on an ELISA plate (Sigma-Aldrich, United States). The plate was treated according to the manufacturer's protocol, and it was read at 450 nm. Thereafter, concentrations were extrapolated from the standard curve.

4.3.5 Statistical analysis

For each study, three independent experiments were performed, unless stated otherwise. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option. A *p*-value of less than or equal to 0.05 was

considered significant. To this end, a value with a different alphabet to the other implies there is a significant difference while those with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p*-value less than 0.05 indicated abnormal distribution of the data points.

4.4 RESULTS

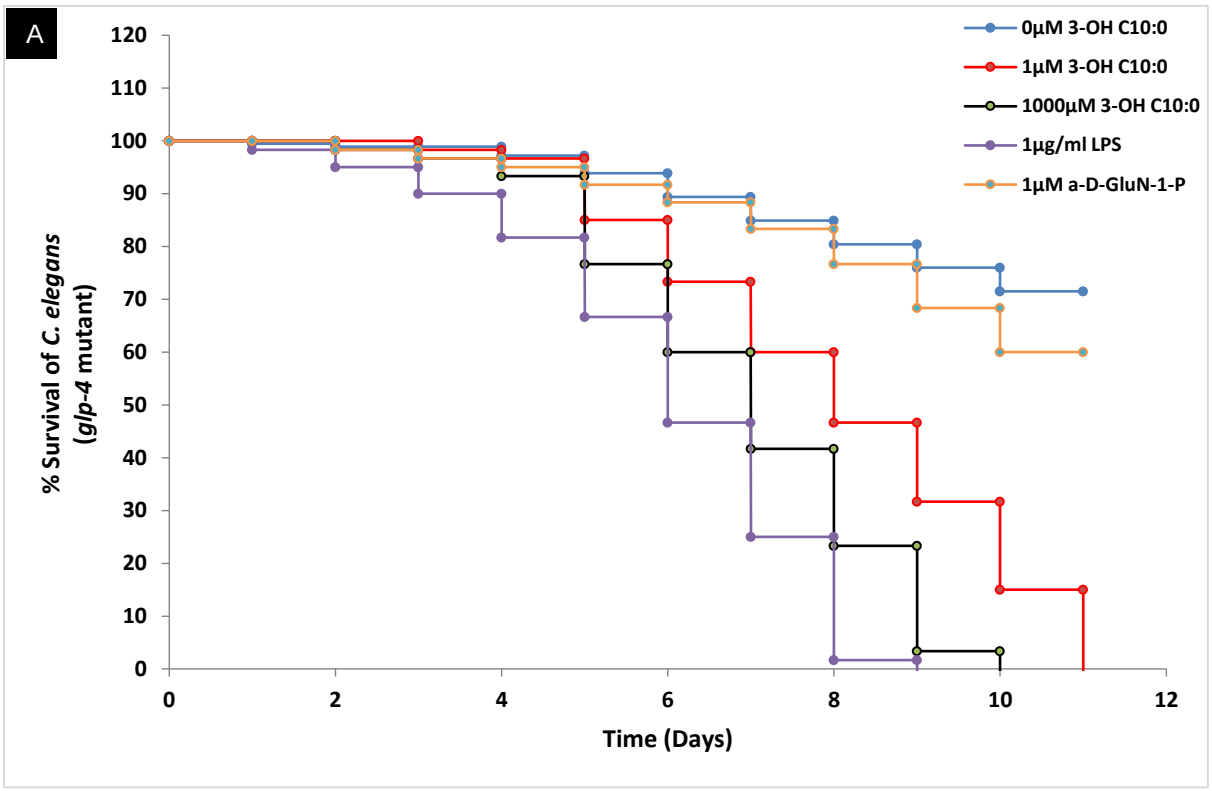
4.4.1 3-OH C10:0 impact on the nematodes

4.4.1.1 Survival

The nematode survival assay results are summarised in Fig 1. The analysis of the 11-day data obtained from the non-treated nematodes revealed that both mutants (single and double deletion) maintained a survival rate above 70% on day 11 (time of study termination; Figs 1a and 1b). When compared to the non-treated nematodes, exposure of the single deletion mutant to α-D-glucosamine-1-phosphate decreased its survival rate by 12% ($p > 0.05$) while the same compound decreased the survival rate of the double deletion mutant by 15% ($p > 0.05$).

Exposure to 1 µM of 3-OH C10:0, significantly decreased (when compared to non-treated nematodes; $p < 0.05$) the survival rate of the nematodes. The survival of both the single and double deletion mutants was reduced to 11 days. While an increase in the 3-OH C10:0 concentration to 1000 µM was observed to affect the survival of the single deletion mutant to 10 days, and that of the double deletion mutant to 9 days. This seemed to suggest that the immunocompromised mutant was more susceptible to increased concentrations of 3-OH C10:0. At the same time, it was noted that the

LPS was more potent of all the compounds as it limited the survival of the single deletion mutant to 9 days and that of the double deletion mutant to 8 days.



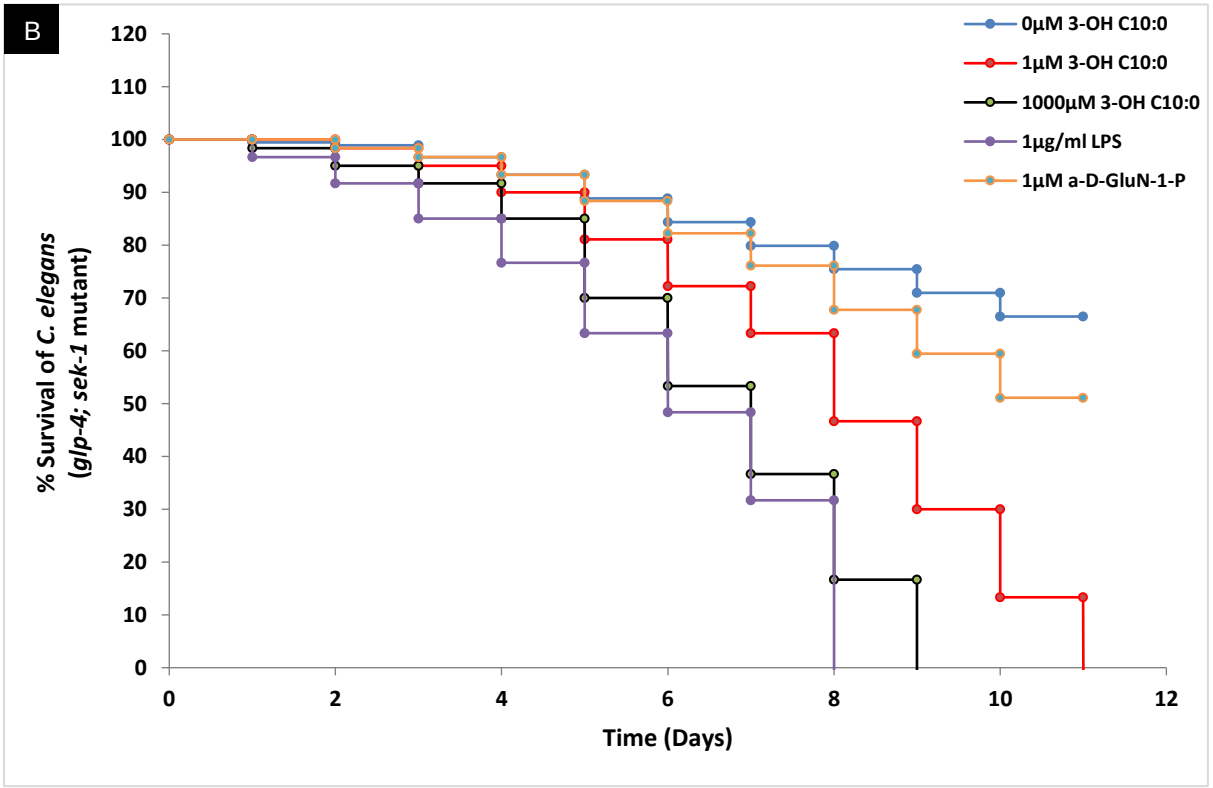


Fig 1. Plots of Kaplan-Meier product estimates of survival of the nematodes after being treated with different test compounds. (**A**) depicts the response of the single deletion Δ(*glp-4*) mutant while (**B**) shows the response of the double deletion Δ(*glp-4*) Δ(*sek-1*) mutant. The non-treated nematodes were used as a reference in each plot. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-p = α-D-glucosamine-1-phosphate.

4.4.1.2 Physiology

To evaluate the effect of 3-OH C10:0 on the physiology of the nematodes, MAPK p38 (Fig. 2) and ERK 1/2 (Fig. 3) were targeted. When considering the results of the single deletion mutant when treated with 1 μM in comparison to the non-treated nematodes (summarised in Fig 2a and b), it was noted the MAPK p38 phosphorylation levels of the treated nematodes yielded a numerical output of -5% at 6 h and +8% at 24 h, respectively. However, upon calculating the *p*-values, it was noted that these levels, at these time intervals, were comparable ($p > 0.05$) to that of non-treated nematodes. This was also true for the α-D-glucosamine-1-phosphate. However, an increase in the 3-OH C10:0 concentration to 1000 μM as well as exposure to the LPS, led to a significant ($p < 0.05$) increase in the phosphorylation levels of MAPK p38. This increase caused by the LPS was to be expected since it is a pro-inflammatory compound that is known to stimulate the MAPK p38 pathway (Han *et al*., 1994). It also seems as though 3-OH C10:0 may have a pro-inflammatory quality when used at a high concentration of 1000 μM.

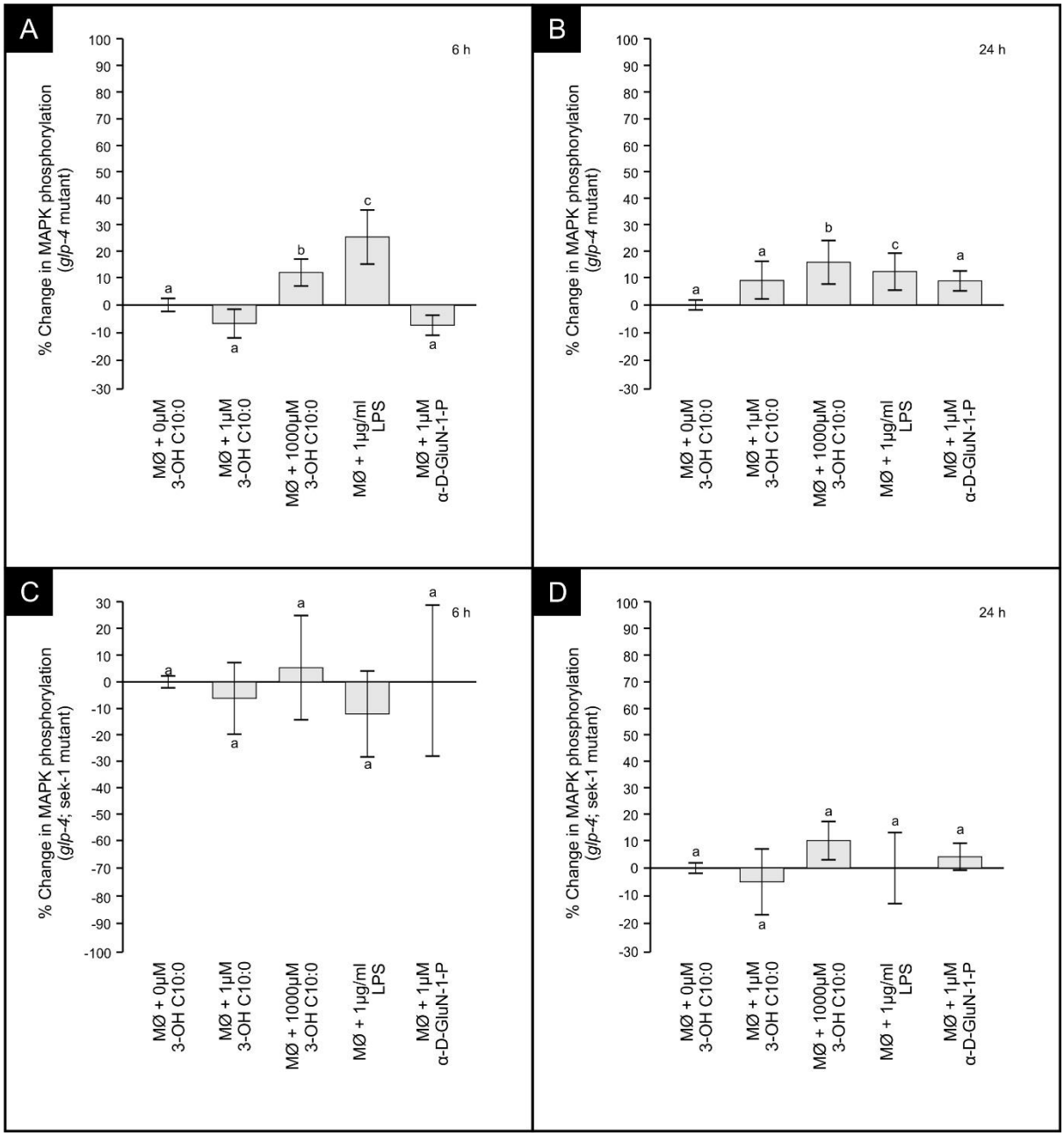


Fig 2. The effect of different compounds on the activation of the MAPK p38 pathway. (**A**) Summarises the results obtained for the single deletion Δ(*glp-4*) mutant after 6 h of incubation, while (**B**) details the results after 24 h. (**C**) Summarises the results obtained of the double deletion Δ(*glp-4*) Δ(*sek-1*) mutant, while (**D**) details the results after 24 h. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b; c] = Significantly different ($p < 0.05$).

Concerning the double deletion mutant, the *sek-1* gene product is reported to be crucial in the activation of the MAPK p38 pathway (Kim *et al*., 2002), which was targeted in the assay. Thus, the deletion of *sek-1*, would, in turn, impair the MAPK p38 pathway. It was, therefore, not surprising to note there was no significant difference between the data of the non-treated nematodes, when compared to that of 3-OH C10:0, the α-D-glucosamine-1-phosphate or the LPS (Fig. 2c and d). When comparing the response of the single deletion mutant to the double deletion mutant, it is clear that the single deletion mutant was more responsive to the presence of 3-OH C10:0; especially at 1000 µM concentration.

When analysing the ERK1/2 data, summarised in Fig. 3, it was noted that the single deletion mutant (which is immunocompetent) was not negatively ($p > 0.05$) affected by the different compounds when compared to the non-treated nematodes. However, the same was not true for the immunocompromised, double deletion mutant. Although the α-D-glucosamine-1-phosphate did not signal for processes that may affect nematode growth and development, it was clear that 3-OH C10:0 (at both concentrations and at 24 h) was able to activate the ERK 1/2 pathway. Moreover, the LPS was the only compound to activate the ERK 1/2 pathway as early as 6 h and after 24 h.

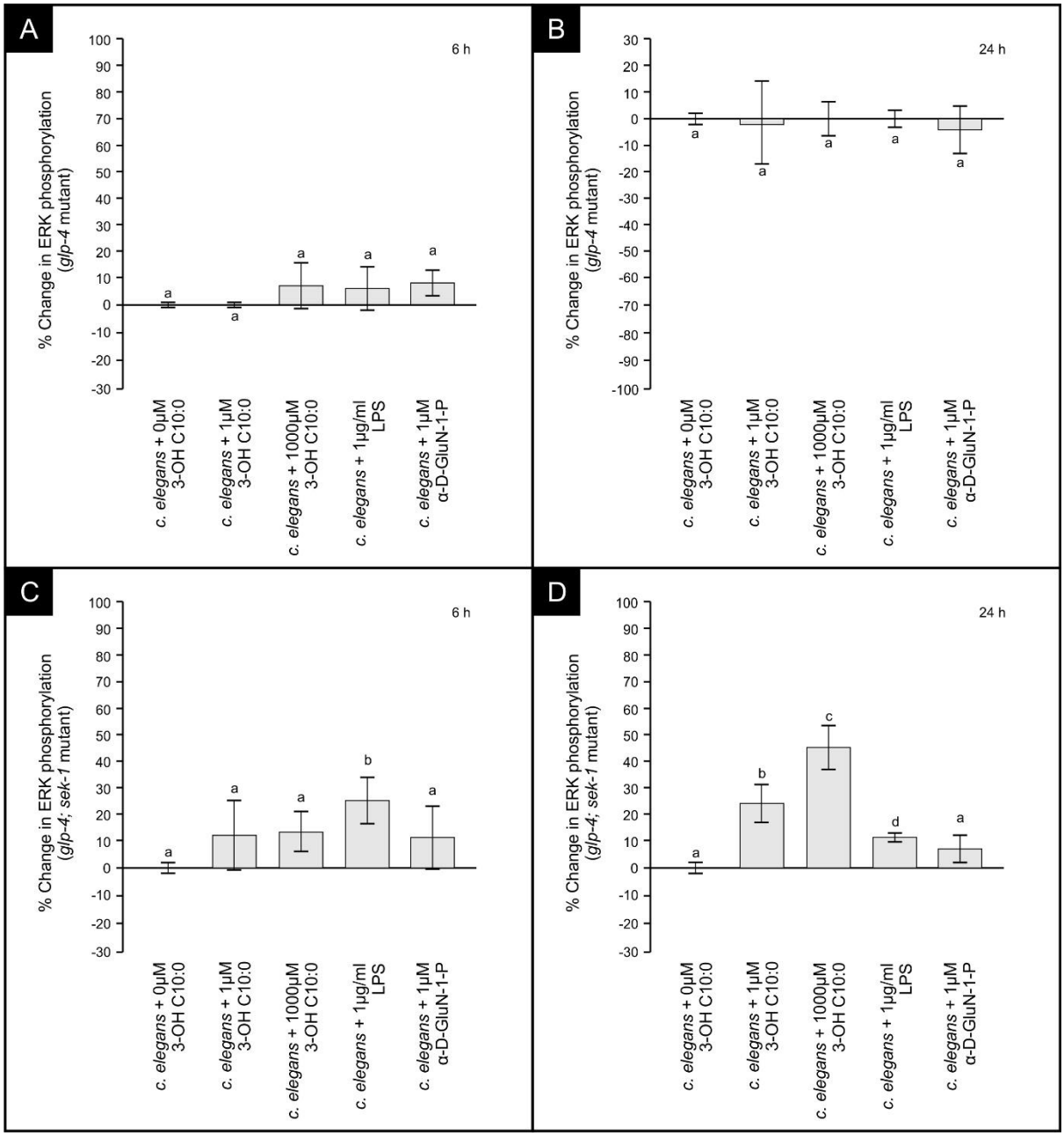


Fig 3. The effect of different compounds on the activation of ERK 1/2 proteins. (**A**) Summarises the results obtained for the single deletion Δ(*glp-4*) mutant after 6 h of incubation, while (**B**) details the results after 24 h. (**C**) Summarises the results obtained of the double deletion Δ(*glp-4*) Δ(*sek-1*) mutant, while (**D**) details the results after 24 h. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b; c; d] = Significantly different ($p < 0.05$).

When comparing the response of single deletion mutant to the double deletion mutant, it is clear that the single deletion mutant was more resistant to stimulation by 3-OH C10:0. Furthermore, when comparing the stimulation of MAPK p38 pathway to that of ERK 1/2 pathway in the single deletion mutant, it is also clear that the MAPK p38 pathway was more responsive to the presence of 3-OH C10:0. This may, in part, explain the survival assay results (Fig. 1); where 3-OH C10:0 was observed to affect the survival of the challenged nematodes. In nematodes, the MAPK p38 pathway is required for the control of pathogen response, including microbial factors and stress response (Li *et al*., 2018).

4.4.2 3-OH C10:0 effect on laboratory rats

4.4.2.1 Weight

The laboratory rats were able to tolerate the procedure of injection, this was evident since no animal died during the study. Moreover, there were no observable differences in the appearance of all the rats, the quality of the coats and texture remained the same. However, the indicator is subjective and given the short duration of the study (24 h), perhaps the time was not sufficient to note hair loss. Nonetheless, the time seemed to be enough for the animals to react to the injected compounds. Only animals that were challenged with 1000 µM of 3-OH C10:0 (Group III) and 1 µg/ml of the LPS (Group IV) displayed a significant ($p < 0.05$) weight loss of 4% (Group III) and 5% (Group IV), respectively (Fig. 4). Weight loss was observed in both sexes.

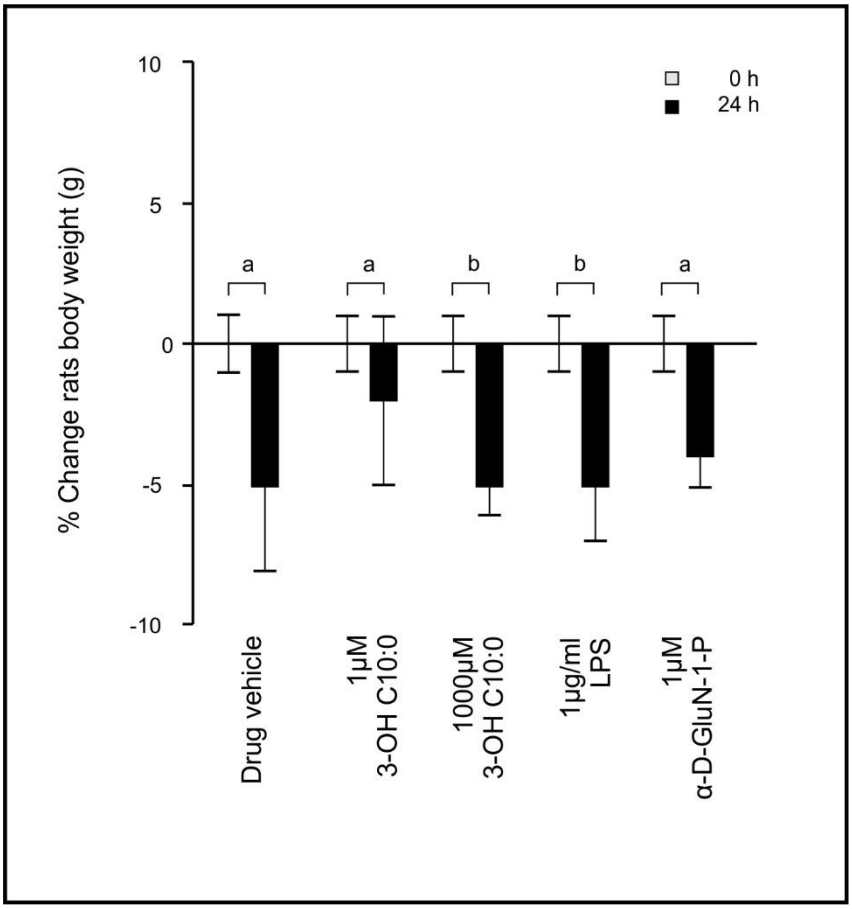


Fig 4. The effect of different concentrations on animal weight loss; used as an indicator of physical stress. Weight measurements of laboratory rats were recorded at the start and termination of the study. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b] = Significantly different ($p < 0.05$).

4.4.2.2 3-OH C10:0 elevated the numbers of monocytes in the blood and stimulated the production of IFN-γ

The presence of the 3-OH C10:0 in the blood seemed to significantly ($p < 0.05$) stimulate the production of monocytes (Fig. 5) within 6 h of exposure. This was true for 3-OH C10:0 at both concentrations. A similar response was observed for the LPS ($p < 0.05$), while the presence of the α-D-glucosamine-1-phosphate in the blood did not lead to elevated levels ($p > 0.05$) of monocytes. After 24 h, numbers of the monocytes subsided for all tested animals.

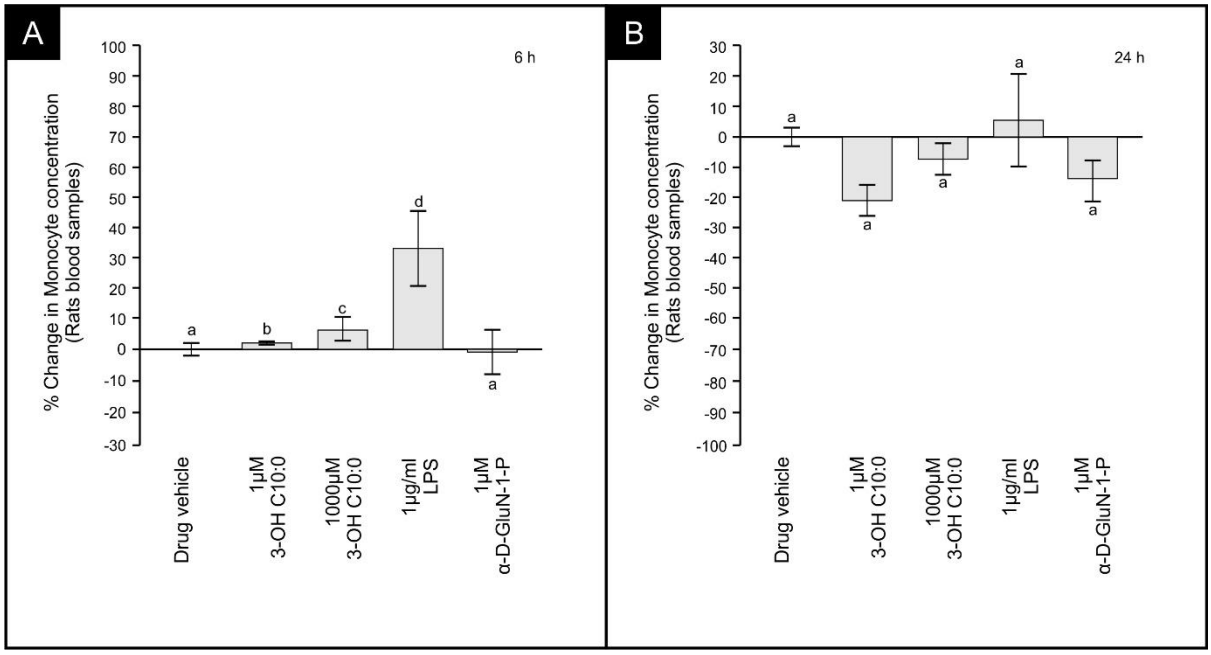


Fig 5. The effect of different compounds on the levels of monocytes detected in the rat's blood samples. Blood sampling was performed after (**A**) 6 and (**B**) 24 h post compound injection. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b; c; d] = Significantly different ($p < 0.05$).

The IFN-γ results are summarised in Fig. 6. The data revealed that at 6 h (Fig. 6a), there was no significant ($p > 0.05$) difference in the levels of IFN-γ when the animals were challenged with 1 µM of 3-OH C10:0 (Group II; -5%) and the α-D-glucosamine-1-phosphate (Group V; +6%), when compared to rats challenged with the drug vehicle (Group I). However, the exposure of animals to a higher concentration (1000 µM) of 3-OH C10:0 (Group III) and the LPS (Group IV) led to significantly elevated levels of IFN-γ. After 24 h (Fig. 6b), all the Groups (II, III, IV, V) animals were not stimulated sufficiently to produce elevated levels of IFN-γ. It may be possible that after 24 h, these compounds were eliminated from the animals.

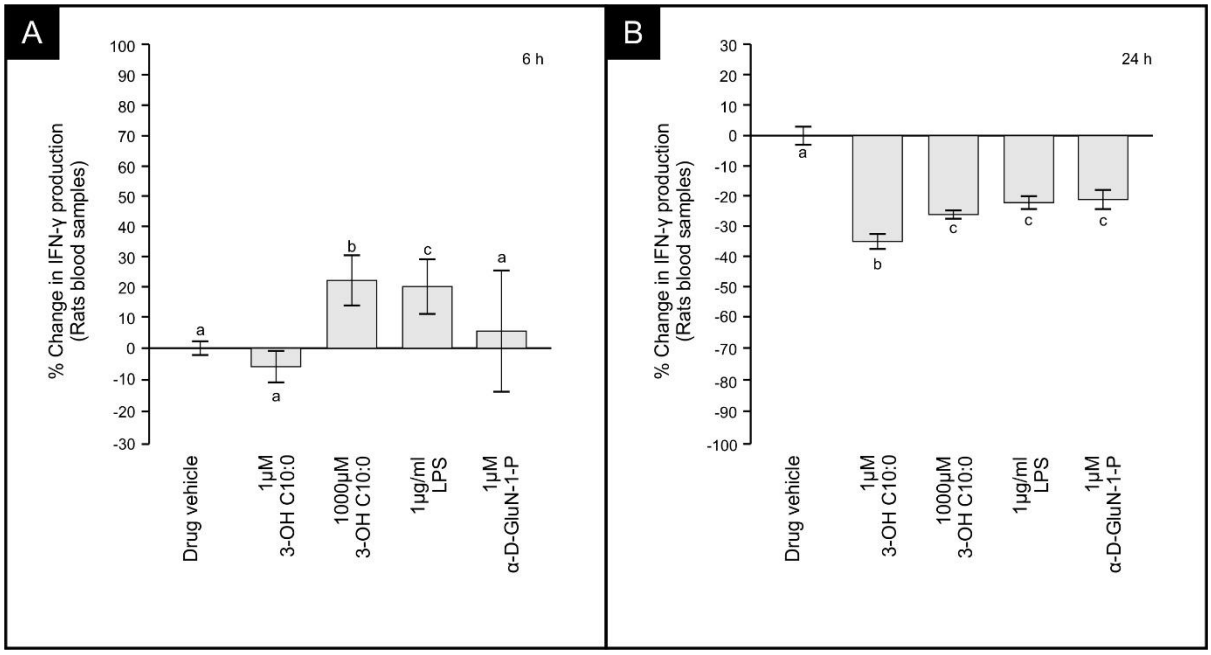


Fig 6. The effect of different compounds on the production of IFN-γ. Blood sampling was performed after (**A**) 6 and (**B**) 24 h post compound injection. 3-OH C10:0 = 3-hydroxydecanoic acid. LPS = Lipopolysaccharides. α-D-GluN-1-P = α-D-glucosamine-1-phosphate. [a] = Not significantly different ($p > 0.05$); [b; c] = Significantly different ($p < 0.05$).

When Figs 5 and 6 are considered together, it is clear that the number of circulating monocytes is linked to the levels of IFN-γ as monocytes play a role in producing this cytokine during inflammation (Serbina *et al*., 2008; Kak *et al*., 2018). The preliminary data of the rats that is documented in the study suggests that 3-OH C10:0 similar to the LPS, may have a pro-inflammatory quality. However, it is could be interesting to include a concentration gradient to gain insight into the dose-dependent response and to identify breakpoints in the prepared range.

4.5 DISCUSSION

The combinational application of multiple models has been reported to be crucial for the accurate interpretation of pathogen-host interactions, including the role of microbial factors (Lange *et al*., 1995; Buras *et al*., 2005; Jiminez *et al*., 2015). In the current study, invertebrate and vertebrate models were used to assess the responses of these organisms to 3-OH C10:0. Firstly, it was demonstrated that 3-OH C10:0 could limit the survival of both the immunocompetent (single deletion mutant; Δ(*glp-4*)) and immunocompromised (double deletion mutant; Δ(*glp-4*) Δ(*sek-1*)) nematodes. Interestingly, Cabreiro and Gems reported that bacterial LPS and the O-antigen could regulate the lifespan of nematodes by increasing bacterial colonisation and shortening nematode lifespan (Cabreiro and Gems, 2013). A study by Maier et al. (2010) demonstrated the effect of structural differences in bacterial LPS, they found that the differences in the B and K-12 strains of *E. coli* were able to extend or reduce the lifespan of *C. elegans*. Kutschera et al. (2019) had argued that the immunomodulatory centre of the LPS may reside in the 3-OH fatty acids. To this end, it was not surprising to see 3-OH C10:0 (at 1 µM and 1000 µM) similarly limiting the survival of the

nematodes. The reduction in the survival rate may further be attributed to a patho-biological process(es) that may have been induced by the presence of 3-OH C10:0. This statement is based on the obtained response profile of the single deletion mutant MAPK p38 pathway towards 3-OH C10:0. From literature, it is clear that the MAPK p38 pathway is stimulated for the control of a pathogen response (including microbial factors), stress and cytokines (Peyssonnaux and Eychène, 2001; Millet and Ewbank, 2004; Troemel *et al*., 2006). Based on the immune status of the nematodes used in the study, it is reasonable to conclude that 3-OH C10:0 impacted the MAPK p38 more than the ERK pathway. This conclusion is based on assessing the response of the single deletion nematodes towards this fatty acid (Figs 2a and 3a). The determination that this fatty acid impacted more the MAPK p38 pathway implies it may be more of a pro-inflammatory compound.

In their paper, Kutschera reported that animals might sense various hydroxy fatty acids proteins through G protein-coupled receptors (Kutschera *et al*., 2019). To the point, the authors documented that the pro-inflammatory receptor GPR84 expressed in leukocytes, like monocytes, can sense 3-hydroxy fatty acids. In this study, the presence of 3-OH C10:0 in laboratory rats seem to have signalled for the recruitment of monocytes and the production of IFN-γ. This may, in turn, signal for further immunological development to control 3-OH C10:0-induced patho-biological process(es). And as shown in Chapter 3 regarding the effect of this molecule on macrophages, it is possible that 3-OH C10:0 may lead to the production INF-γ. In conclusion, these findings may be relevant to understanding the response of a host that has been exposed to 1 µM of 3-OH C10:0, a concentration that is estimated to be produced by 1 x 10^8 pseudomonal cells.

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CHAPTER 5

General Discussion

5.1 DISCUSSION

The studies detailed herein were the first, formative work that shed light into the role of pseudomonal 3-hydroxy fatty acids particularly 3-hydroxydecanoic acid (3-OH C10:0) in the context of an animal cellular response to exposure. This information allows for an expansion of our understanding into the function of these molecules, more so when produced by bacteria.

It was reasoned that these molecules were secreted in free-form based on the extraction protocol. This also supports the idea that the detected 3-OH C10:0 was not cleaved from the lipopolysaccharide (LPS) but, it was purposely derived from one of the other biosynthetic pathways highlighted in the in the literature review section. In chapter 2, only saturated 3-hydroxy fatty acids were detected. This is primarily because of the analytical standards that were chosen. It is possible that if different standards were chosen, other species (unsaturated, branched, etc.) may have been detected, including other hydroxy fatty acids. More importantly, these undetected hydroxy fatty acids species may also play a role in the pathogenesis of *P. aeruginosa*.

It would be interesting to determine if pseudomonal cells could scavenge host lipids during an infection to produce 3-hydroxy fatty acids. Other studies have shown that a molecule such as arachidonic acid can be bio-transformed by fungal species to produce a number of 3-hydroxy fatty acid species (Pohl *et al*., 1998; Ciccoli *et al*., 2005). And, in the case of *P. aeruginosa*, arachidonic acid can be converted to other hydroxy fatty acids, i.e. 15-hydroxy eicosatetraenoic acid (15-HETE), which is a potent immune response modulator (Vance et al. 2004). It would also be interesting to

determine whether these molecules, following production, act alone, associate with cellular structures or incorporate into other molecules. In the case of pseudomonal cells, free-form 3-hydroxy fatty acids can also be incorporated into rhamnolipids (Rahim *et al*., 2001; Soberón-Chávez *et al*., 2005; Abdel-Mawgoud *et al*., 2010), which are also a virulence determinant that can impede phagocytosis (McClure and Schiller, 1992, 1996; Jensen *et al*., 2007).

Unlike in humans, microbial 3-hydroxy fatty acids seem to be purposed to have a secondary function in support of the microbe. And in some cases, as documented by this study, they may be implicated in pathogenesis. The idea that lipids can influence patho-biological processes is now generally accepted. Noverr et al. (2003) showed that microbial prostaglandins could function in the modulation of chemokines and the production of tumour necrosis factor alpha. Therefore, these compounds assist in manipulating infectious processes that may manifest a diseased state (Noverr *et al*., 2003). Regarding the results from this current study, it was observed that 3-OH C10:0 and the LPS were able to induce the activation of mitogen-activated protein kinase (MAPK p38) and increased production of interferon-gamma (IFN-γ) by macrophages. Interferon-gamma produced by macrophages has been implicated in early host response to pathogens (Kak *et al*., 2018), therefore, it is possible that the macrophages recognised 3-OH C10:0 as a microbial agent worthy of an immune response similar to the LPS.

There is evidence that shows G protein-coupled receptors are responsible for sensing these molecules in the extracellular environment. As shown by the Nigam et al. (1999) and Kutschera et al. (2019) studies, the detection of low-complexity microbial

metabolites is the beginning stage of modulating host cellular responses in favour of the microbe. While this was not the focus of this study, it is reasonable to conclude that 3-OH C10:0 may have been sensed by G protein-coupled receptors. Ultimately, it was possible for this 3-hydroxy fatty acid to influence macrophage function, and the biology of laboratory nematodes as well as rats. Regarding the observed inflammatory response, 3-OH C10:0 acted in a similar manner to the LPS. Again, this may be due to the idea posited by Kutschera et al. (2019) that a host organism does not sense large molecules (like the LPS) rather they sense low-complexity bacterial metabolites such as the presence of 3-OH C10:0 in the LPS, to trigger an immune response. In fact, these authors argued that 3-hydroxy fatty acids were a key structural feature for LORE-dependent lipopolysaccharide sensing. An interesting function of these molecules was their anti-phagocytic quality. This may be due to their incorporation into the lipid phase of macrophage cell membrane; affecting phagocytosis as argued by Schroit and Gallily (1979), preventing the release of lysozymes (Eftimiadi *et al*., 1987) or reducing hydrogen peroxide production thus impairing phagocytosis (Bellinati-Pires *et al*., 1993). Nonetheless, further studies are required to understand the molecular mechanism(s) that confer the protective quality of 3-hydroxy fatty acids.

One shortcoming of the designed studies was the lack of a concentration gradient that included more test concentrations. This addition would help in the identification of: (1) dose-dependent trends, and (2) breakpoints in the response of host cells or whole organism to the 3-OH C10:0. However, the effect of 3-OH C10:0 at the estimated physiological concentration (obtained from 10 million cells) cannot be overlooked. Future studies should also consider the use of pseudomonal cells with a mutation defect (and corresponding complemented cells) in one of the biosynthetic pathways

of 3-hydroxy fatty acids. This would assist in pinpointing the central biosynthetic route. The use of chemical drugs that are similar in structure to enzymes that catalyse the biosynthetic routes should also be considered. The action of the drugs should also be assessed in combined therapy with traditional antibiotics.

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