Investigation into the effect of fatty acids on the yield and replication of rotavirus in cell culture

By

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ABBREVIATIONS

aa:	amino acid
AA:	arachidonic acid
ADRP:	adipose differentiation-related protein
bp:	base pairs
CHIKV:	Chikungunya virus
CLSM	confocal laser scanning microscopy
CM:	confocal microscopy
CoA:	coenzyme A
COX:	cyclooxygenase
$cPLA_2$:	cytoplasmic phospholipase A_2
DENV:	Dengue virus
DG:	diacylglycerol
DGAT:	diacylglycerol acyltransferase
DLP:	double-layered particle
DMEM:	Dulbecco's Modified Eagle's medium
EDAC:	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
eLDs:	expanding lipid droplets
ELISA	enzyme-linked immunosorbent assay
ER:	endoplasmic reticulum
ERK:	extracellular signal-regulated kinase
FA:	fatty acid
FIT:	fat-storage and-inducing transmembrane
GLA:	γ-linolenic acid
HCV:	hepatitis C virus
Huh-7:	human hepatoma cells
IB:	inclusion bodies
IFN:	interferon
iLDs:	initial lipid droplets
LDs:	lipid droplets
LM:	light microscopy
MA104:	monkey kidney cells
MAVS:	viral antagonist of mitochondrial antiviral-signalling protein

MGAT:	monoacylglycerol O-acyltransferase
MT:	microtubule
NEAA:	non-essential amino acid
NSP:	non-structural protein
OA:	oleic acid
ORF:	open reading frame
PA:	phosphatidic acid
PABP:	poly A binding protein
PBS:	phosphate buffer saline
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PI:	phosphatidylinositol
PKA:	protein kinase A
PLA_2:	phospholipase A_2
PLD:	phospholipase
PLIN:	perilipin
PUFAs:	poly-unsaturated fatty acids
RdRP:	RNA-dependent RNA polymerase
ROS:	reactive oxygen species
RRV:	rhesus rotavirus
RV:	rotavirus
SA:	stearic acid
SA11:	simian rotavirus strain 11
SAM:	S-adenosyl-L-methionine
SLP:	single-layered particle
TAGs:	triacylglycerols
TCs:	transcription complexes
Th:	T helper cell class
TIP-47:	tail-interacting protein 47
TLP:	triple-layered particle
VLP:	virus-like particles
VLS:	viroplasm-like structures
VP:	structural viral protein

SUMMARY

Rotavirus (RV) remains one of the leading causes of severe dehydrating diarrhoea in infants and young children. The successful replication of RV relies on the formation of viroplasms, which consist of several viral proteins and host lipid droplets (LDs). Several studies have indicated that the absence of LDs or fatty acids (FAs) for the formation of LDs prevent the formation of viroplasms and subsequently severely hamper RV replication. Lipid droplets are well-known for their storage of fatty acids and their downstream metabolites that can play roles in immunological response toward invading pathogens. Furthermore, the type of FAs within LDs plays critical roles in shaping both the type and strength of immune responses.

This study sought to determine the effects that supplementation of MA104 cells with FAs of varying saturation could have on RV replication. The effects of supplementing MA104 cells with albumin (as control), stearic acid (SA, 18:0), oleic acid (OA, 18:1) and γ-linolenic acid (GLA, 18:2) on the lipid profile of M104 cells were determined using gas chromatography. Results indicated that each supplementation was able to change the lipid profile of MA104 cells in unique ways. Albumin supplementation showed no significant difference from the unsupplemented and uninfected control, while SA supplementation appeared to lower/increase some FAs, but not itself, when compared to unsupplemented and uninfected control. Oleic acid supplementation increased the relative percentage of itself, while GLA supplementation increased both the relative percentage of itself and arachidonic acid (AA) compared to the unsupplemented and uninfected control. The subsequent infection of supplemented MA104 cells further modulated the lipid profile of MA104 cells. Infection, of cells supplemented with GLA, showed an increase in the relative percentage of both GLA and AA when compared to the supplemented uninfected control. Interestingly, the study found that RV decreased the amount of GLA and AA in cells supplemented with GLA, possibly indicating that the metabolism of these FAs is driven to produce prostaglandin E_2 (PGE_2), a well-known modulator for immunity.

The use of tissue culture infection doses 50 ($TCID_{50}$) showed that the supplementation of M104 cells with unsaturated FAs (OA and GLA) increased the rate of RV replication when compared to the unsupplemented and infected control. In particular, the supplementation of GLA showed to be an effective enhancer of RV replication. In contrast, supplementation with albumin and saturated FAs (SA) showed no significance in rate of replication when compared to the unsupplemented and uninfected controls.

Detection of PGE_2 using ELISA showed that RV can increase the production of PGE_2 regardless of supplementation. However, the supplementation of MA104 cells with GLA led to a 2-fold increase in the amount of PGE_2 compared to unsupplemented and infected MA104 cells. By using confocal laser scanning microscopy, it was shown that LDs, PGE_2 and NSP2

(viral protein present in viroplasms) co-localized in supplemented and uninfected MA104 cells. Co-localization between NSP2 and PGE_2 was also observed for the first time during the study indicating a possible role for viroplasms during the increased production of PGE_2.

Together, results from this study shows that unsaturated FAs (OA and GLA) can increase the replication of RV. Although the exact mechanism behind this effect remains unknown, data indicates that there could be a role for PGE_2. Thus, by elucidating the mechanism by which unsaturated FAs (and possibly PGE_2) increase the replication rate of RV, novel anti-viral strategies against RV could be developed.

Key terms

Tissue culture, rotavirus, rotavirus SA11 strain, fatty acids, stearic acid, oleic acid, γ-linolenic acid, prostaglandin E_2, viral infection, viral immunity, viral replication

- Conferences:

    Sander, W.J., Pohl, C.H., O'Neill H.G. Investigation into the effect of fatty acids on the yield of rotavirus infection. **South African Society for Microbiology 2016 Conference,** Durban, 17 - 20 January 2016 [Poster].

    Sander, W.J., Pohl, C.H., O'Neill H.G. The effect of supplementation of fatty acids with varying degrees of saturation on rotavirus yield and replication in MA104 cells. **South African Society of Biochemistry and Molecular Biology 2018 Conference,** Potchefstroom, 8 - 11 July 2018 [Poster].

    Sander, W.J., Pohl, C.H., O'Neill H.G. The effect of γ-linolenic acid supplementation on rotavirus yield and replication in MA104 cells. **13th International dsRNA virus symposium**, Houffalize, Belgium, 24 – 28 September 2018 [Poster].

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- Publications:

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CHAPTER 1: THE ROTAVIRUS AND LIPID DROPLET CONNECTION

1.1. Introduction

Rotavirus (RV) forms part of the double-stranded RNA (dsRNA) virus family, *Reoviridae*. As one of the leading causes of acute gastroenteritis in infants and young children (Estes and Greenberg, 2013), RV is estimated to be responsible for approximately 128 500 deaths per year in this age group (Troeger et al., 2018). Mammals (mainly mice, monkeys, cattle and bats) and birds have so far been identified as major hosts for RV (Dubovi and Maclachlan, 2011; Estes and Greenberg, 2013).

Rotavirus is a non-enveloped virus with an icosahedral structure and is transmitted via the faecal-oral route (Bishop et al., 1973; Flewett et al., 1973). Rotavirus has six structural proteins (VPs) and six non-structural proteins (NSPs) which are encoded by its 11 genome segments (Estes and Greenberg, 2013). Rotavirus consist of three layers, with the outer layer having spike-like protrusions. Currently there are eight serogroups of rotavirus, with groups A – C and H responsible for disease in mammals, while groups D, F and G have mainly been found to infect avian species with group E infecting pigs (Kindler et al., 2013; Martella et al., 2010; Matthijnssens and Van Ranst, 2012). Group A is the most prevalent cause of diarrhoea in young children and infants. Group A RV is thus of critical importance to human health and is to date the most thoroughly studied RV group (Desselberger, 2014). Binary genotyping in rotavirus relies upon the genetic relatedness of the partial nucleotide sequences of genome segment 9, encoding VP7 (G-types) and genome 4, encoding VP4 (P-types) (Estes and Greenberg, 2013).

Cells infected with RV form characteristic cytoplasmic inclusion bodies called viroplasms (Fabbretti et al., 1999), which result from the co-expression of certain VPs and NSPs and their aggregation with lipid droplets (LDFAs) (Cheung et al., 2010). Replication and packaging of the RV genome into capsid intermediates occur within viroplasms (Desselberger, 2014). RV virions are formed when these capsid intermediates migrate and bud into the endoplasmic reticulum (ER) (Taylor et al., 1996). Lipid droplets are used as a scaffold for viroplasm formation (Cheung et al., 2010; Lever and Desselberger, 2015). Recently, classified as organelles, LDs are known to be at the centre lipid and energy metabolism (Guo et al., 2009). Lipid droplets can be classified based on the composition of lipids and proteins and are known to interact with cellular organelles. Lipid droplets are also major hubs for the production of eicosanoids since arachidonic acid (precursor to eicosanoids) and the major eicosanoid synthesizing enzymes have been found to localize at LDs (Meester et al., 2011). Eicosanoids are signal molecules responsible for the modulation of immunity (Esser-von Bieren, 2017).

Work done by Cheung and co-workers (2010) on inhibiting both the synthesis of fatty acids (FA) and the formation of lipid droplets showed a decrease in viral progeny. In this review, the current knowledge of the interaction and role of LDs with viroplasms will be reviewed. The

importance of LDs and eicosanoids in RV replication will be highlighted as well as the possibility of targeting LDs as potential therapeutic agents.

1.2. Rotavirus

1.2.1. Structure and genome

1.2.1.1. Structure

Rotavirus (RV) obtained its name due to its' wheel with short spokes of a well-defined rim morphological appearance (Flewett et al., 1974). Within the centre of the RV virion are replication complexes (VP1 and VP3), around which it is suggested the genomic RNA segments are arranged in conical cylinders, but details regarding the structure within the core have only been explored in the last decade (Lu et al., 2008). The replication complexes and RNA are surrounded by 60 dimers of VP2 and this results in the single-layered particle (SLP, also known as the core shell) (McClain et al., 2010). The SLP is in turn surrounded by 260 trimers of VP6 which form the double-layered particles (DLPs) (Prasad et al., 1988). Finally, the triple-layered particle (TLP) is formed when trimers of VP4 and VP7 surround the DLP. Three types of channels are spread throughout the structure of RV, namely type I, type II, type III channels (Lu et al., 2008). The classification of these channels is due to their position and size, but their function remains unknown. It has been proposed that the channels allow for the importing and exporting of metabolites and RNA transcripts, respectively (Prasad et al., 1988).

1.2.1.2. Gene coding assignments

The genome of RV consists of 11 segments of dsRNA (Mertens et al., 2003). The entire genome of RV differs among strains but the genome of SA11 (simian rotavirus strain) consist of approximately 18 500 base pairs (bp) with the segments ranging from 670 to 3 302 bp (Mlera et al., 2013). In **Table 1. 1** the genome segments encoding structural (VP1 to VP4, VP5*, VP6, VP7 and VP8*) and non-structural (NSP1 to NSP6) proteins are shown. The viral particle consists of structural proteins (VP) while the non-structural proteins (NSP) are only found during viral replication and within non-mature viral particles (Prasad et al., 1988; Yeager, 1990).

Table 1. 1. Summary of the genomes, coding assignments and protein information of rotavirus. Genome segment sizes of the prototype rotavirus SA11 are provided (Mlera et al., 2013)

Genome segment	Size (bp)	Encoded Protein	Size (kDa)	Location	Functions	References
1	3302	VP1	125	Inner capsid	RNA-dependent RNA polymerase; ssRNA binding; transcription complex	Burns et al., 1996; Patton, 1996, 2001; Patton et al., 1997; Prasad et al., 1996; Zeng et al., 1996)
2	2683	VP2	102	Inner capsid	Core shell; RNA binding; required for RNA-dependent RNA polymerase activity	Berois et al., 2003; Bican et al., 1982; Boyle and Holmes, 1986; Clark and Desselberger, 1988; Mitchell and Both, 1990a; Patton et al., 1997; Zeng et al., 1994
3	2591	VP3	98	Inner capsid	Guanyltransferase; methyltransferase; 2',5'-phoshodieasterase; ssRNA binding; transcription complex, MAVS antagonist	Burns et al., 1996; Chen et al., 1999; Ding et al., 2018; Liu et al., 1992; Liu and Estes, 1989; Patton, 2001; Pizarro et al., 1991; Prasad et al., 1996
4	2362	VP4[1] VP5* VP8*	87	Outer capsid	P type neutralization antigen; attachment protein; virulence; fusion with cell membrane	Anthony et al., 1991; Denisova et al., 1999; Dormitzer et al., 2002b; Ericson et al., 1983; Fiore et al., 1991; Hoshino et al., 1985; Hoshino and Kapikian, 1996; Kalica et al., 1983; López et al., 1985; Ludert et al., 1996; Offit and Blavat, 1986; Ruggeri and Greenberg, 1991; Shaw et al., 1993; Zarate et al., 2000
5	1614	NSP1	59	Non-structural	Interferon antagonist; E3 ligase; RNA binding	Dunn et al., 1994; Graff et al., 2002; Hua et al., 1994; Kojima et al., 1996; Mitchell and Both, 1990b; Patton, 1995, 2001
6	1356	VP6	48	Middle capsid	Protection; required for transcription	Burns et al., 1996; Clark and Desselberger, 1988; Greenberg et al., 1983; Kalica et al., 1981; Mansell et al., 1994; Mason et al., 1980; Prasad et al., 1988; Smith et al., 1989; Tompkins et al., 1975; Yang et al., 2001
7	1105	NSP3	34	Non-structural	Binds to 3' terminus of viral ss (+) RNA; cellular eIF4G; Hsp90, displaces PABP; inhibits host cell translation	Chizhikov and Patton, 2000; Deo et al., 2002; Groft and Burley, 2002; Mattion et al., 1992; Piron, 1998; Poncet et al., 1994; Vende et al., 2000
8	1059	NSP2	37	Non-structural	Binds RNA; NTPase; NDP kinase; helix destabilizing	Afrikanova et al., 1998; Fabbretti et al., 1999; Jayaram et al., 2002; Kattoura et al., 1992, 1994; Patton, 2001; Petrie et al., 1984; Taraporewala et al., 1999, 2002; Taraporewala and Patton, 2001
9	1063	VP7	37	Outer capsid	G type neutralization antigen Membrane penetration	Dormitzer and Greenberg, 1992; Ericson et al., 1982, 1983; Hoshino et al., 1985; Hoshino and Kapikian, 1996; Mason et al., 1980; Michelangeli et al., 1997
10	751	NSP4	20	Non-structural	Interaction with viroplasms and autophagy pathway; modulates intracellular Ca^{2+} and RNA replication; enterotoxin; virulence	Au et al., 1989; Ball et al., 1996; Ericson et al., 1982, 1983; Estes et al., 2001; Jagannath et al., 2000; Meyer et al., 1989; Tian et al., 1994
11	667	NSP5	22	Non-structural	RNA binding; kinase; essential for viroplasm formation	Afrikanova et al., 1996, 1998; Blackhall et al., 1998; González et al., 1998; González and Burrone, 1991; Mattion et al., 1991; Patton, 2001; Poncet et al., 1997
		NSP6	11	Non-structural	Interaction with NSP5 and mitochondria	González et al., 1998; Holloway et al., 2015; Mattion et al., 1991

[1] Cleaved by trypsin or cellular protease into VP5* and VP8*

1.2.1.3. Single- and double-layered particle proteins

It is well known RV single-layered particle (SLP) consists of VP1-3 (Liu et al., 1988). Genome replication (- RNA) and viral mRNA synthesis (+ RNA) is catalysed by RV VP1, a RNA-dependent RNA polymerase (RdRp) (Patton et al., 1997) (**Figure 1. 1**). VP1 is connected to the core of sub-viral particles, and catalysis has been shown to be dependent on the presence of VP2. Four tunnels span the cage-like formation of VP1 that leads to the catalytic cavity (Lu et al., 2008). **Figure 1. 1** shows that VP2 is found within the shell of the SLP and constitutes the majority of the inner capsid (Gonzalez and Affranchino, 1995). Due to the ability of VP2 to bind viral RNA, it assists with viral replication and encapsidation of the viral RNA. In addition to the ability of VP2 to bind viral RNA, it also interacts with VP6 and NSP5 (Berois et al., 2003). The capping of viral mRNA during transcription is achieved by VP3, a guanylyltransferase (Liu et al., 1992; Pizarro et al., 1991). Furthermore, VP3 can bind S-adenosyl-L-methionine (SAM), inferring methyltransferase activity for VP3 (Chen et al., 1999). The internalisation of the viral inner capsid can be selectively mediated by trypsin-cleaved fragments (Fukuhara et al., 1988). Recently, VP3 has been shown to act as a viral antagonist of mitochondrial antiviral-signalling protein (MAVS) by localizing to the mitochondria, where it mediates the phosphorylation of MAVS leading to the subsequent proteasomal degradation and blockade of IFN-γ (Ding et al., 2018).

The double-layered particle (DLP) is formed when VP6 surrounds the outer surface of the SLP. Double-layered particles are responsible for the endogenous transcription of the viral genome (Lawton et al., 2000; Prasad et al., 1988). The VP6 trimer is known to be highly immunogenic and antigenic (Greenberg et al., 1983; Kalica et al., 1981; Sugimoto et al., 2012; Svensson et al., 1988). The formation of tubules and trimerization are an intrinsic property of VP6 (Estes et al., 1987). When VP6 is removed from DLPs, there is a significant loss in transcriptase activity while, similarly, the addition of an excess VP6 to DLPs also results in the loss of transcriptase activity (Arias et al., 1996; Bican et al., 1982). Furthermore, MA104 cells transfected with DLPs, where VP6 was added to SLPs, resulted in infection (Desselberger et al., 2013). Lobeck and co-workers (2016) revealed that rhesus rotavirus (RRV) VP6 induced extracellular signal-regulated kinase (ERK) phosphorylation which lead to an influx of calcium. They concluded that the pathogenesis of RRV-induced murine biliary atresia is dependent on VP6 inducing the phosphorylation of the ERK pathway resulting in an influx of calcium and allowing viral replication in cholangiocytes.
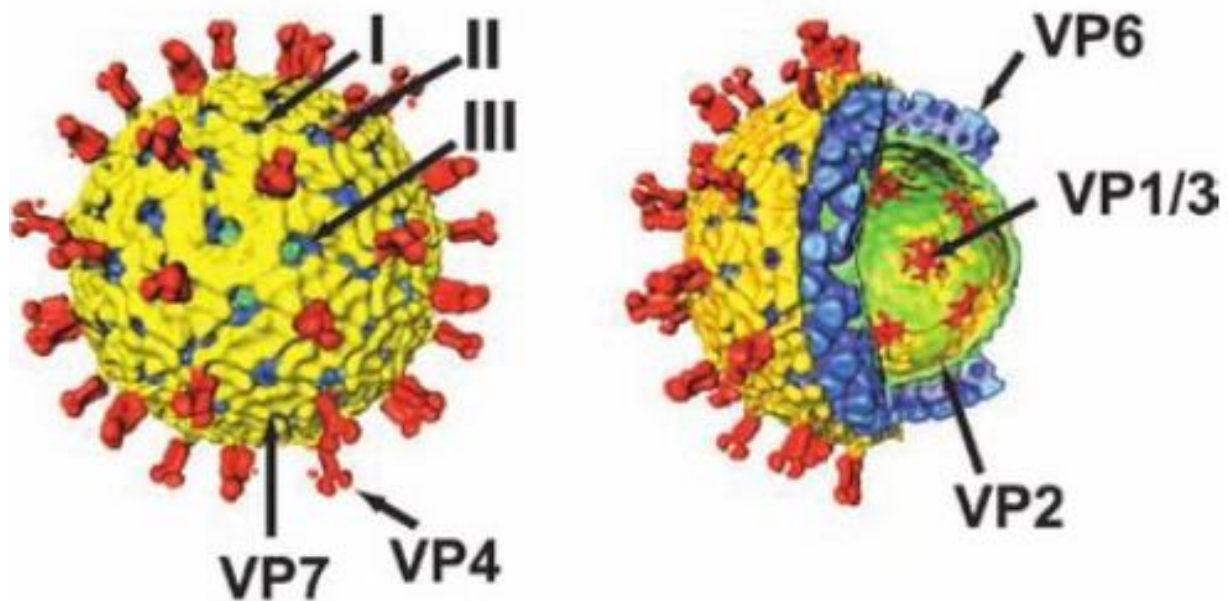
Figure 1. 1. The structure of rotavirus. The model of rotavirus triple-layered particle by cryo-electron microscopy. The transcription enzymes (VP1/VP3) are shown in red and are surrounded by VP2 (green) to form the single layered particle. The double-layered particle of RV with VP6 (inner layer) blue, is surrounded by V7 (outer layer) in yellow with embedded VP4 (spike proteins). The transcriptional enzymes are anchored into VP2. The three types of channels are indicated by arrows (type l, type ll, type lll) (Pesavento et al., 2006).

1.2.1.4. Triple-layered particle proteins

Attachment and penetration by RV is dependent on VP4, while VP4 is also responsible for hemagglutination, neutralization and virulence (Estes and Greenberg, 2013). Viral infectivity is enhanced by the susceptibility of VP4 to proteolysis (Arias et al., 1996). The proteolytic cleavage of VP4 mediates virus entry and results in a several fold increase in viral infectivity. The spike-like protrusions of RV consist of dimers of VP4 (Patton et al., 1993). Li and co-workers (2009) suggested that VP4 extends inward into the type II channels and is anchored to the DLP before being locked into these channels by VP7. Furthermore, their reconstruction of VP4 showed a dimeric appearance above the capsid surface, while having a trimeric appearance below the capsid surface. The cleavage of VP4 by trypsin results in two sub-fragments, namely VP5* and VP8* (Fiore et al., 1991). VP5* mediated cell entry, while VP8* aids in more efficient attachment (Kirkwood et al., 1996; Kobayashi et al., 1990; Padilla-Noriega et al., 1995). VP7 is a glycoprotein and a major component of the outer capsid (Shaw et al., 1993). The protein is highly immunogenic and induces neutralizing antibodies (Hoshino et al., 1985; Matsui et al., 1989). The stability of VP7 trimers are dependent on bound Ca^{2+} (Emslie et al., 1996). Oligosaccharides on VP7 are modified by trimming in the ER (Kabcenell

et al., 1988). VP7 ensures VP5* is kept and stabilized in the upright spike conformation, allowing for attachment by VP8*(Trask et al., 2012).

1.2.1.5.   Non-structural proteins

Due to the lack of structural studies on NSP1, little is known about its role during RV replication (Hu et al., 2012b). It is suggested that NSP1 could act as an antagonist to interferons, while also being responsible for host range (Arnold and Patton, 2011; Bagchi et al., 2010; Graff et al., 2007, 2009). NPS2 plays crucial roles in roles in viroplasm formation (Fabbretti et al., 1999), genome replication/encapsidation and protein interaction with NSP5, VP1 (Valenzuela et al., 1991) and VP2 (Patton et al., 2006), is a multi-functional enzyme. A deep cleft in NSP2 separates nucleotide binding and hydrolysis of phosphate domains (Jayaram et al., 2002; Jiang et al., 2006; Kumar et al., 2007). The activity of NSP2 is dependent on the interaction with ligands, which may enable NSP2 to switch between or use different combinations of catalytic activities (Hu et al., 2012b). The suppression of host protein synthesis is achieved by NSP3, where it acts through antagonism of the poly A binding protein (PABP) (Chung and McCrae, 2011; Keryer-Bibens et al., 2009; Piron, 1998; Poncet et al., 1993; Vende et al., 2000). NSP4 is multifunctional protein with functions that include acting as intracellular receptors for DLPs (Taylor et al., 1996), releasing Ca^{2+} responsible for the stabilisation of TLPs (Hyser et al., 2010; Tian et al., 1996), altering plasma membrane permeability (Newton et al., 1997) and its well-known function as a viral enterotoxin (Ball et al., 1996). Many of these functions have been mapped to distinct domains. In addition, the NSP4 co-localizes with microtubule-associated protein 1A/1B-light chain 3, which together with the increased Ca^{2+} levels, induced the activation of autophagy (Berkova et al., 2006). NSP4 is located in the ER (Taylor et al., 1996) and induces diarrhoea by triggering the secretion of chloride in a calcium-dependent pathway (Ball et al., 1996). The diarrhoea caused by NSP4 is dependent on several factors including age and dose of NSP4. NSP5 exists in several isoforms that range from 28-kDA to 32-kDA for SA11 (Afrikanova et al., 1998). These isoforms are due to the hyperphosphorylation of NSP5 by both VP2 and NSP2. Although the precise mechanism of phosphorylation during infection remains unknown, it is known that both VP2 and NSP2 can hyper phosphorylate NSP5 (Eichwald et al., 2012). The formation of viroplasm-like structures commences when NSP5 interacts with NSP2. In addition, NSP5 can interact with VP1 (Arnoldi et al., 2007), VP2 (Berois et al., 2003) and NSP6 (Poncet et al., 2000). NSP6 has an overlapping reading frame with NSP5 on the 11th genome segment (Mattion et al., 1991). The functions of NSP6 remain unknown, as few studies have explored its structure. NSP6 shows sequence independent nucleic acid binding for ssRNA and dsRNA (Rainsford and McCrae, 2007). Recently, Holloway and co-workers (2015) concluded that when NSP6 is recombinantly

expressed in MA104 cells it localizes with the mitochondria via a predicted N-terminal α-helix. This may suggest that NSP6 could affect the functions and roles of the mitochondria during RV infection. Recently, Komoto and co-workers (2017) revealed that NSP6 is not required for viral replication in cell culture by using the recently developed plasmid only-based reverse genetic system (Kanai et al., 2017).

1.2.2. Replication: An overview

The replication cycle of RV is shown in **Figure 1. 2** and includes the following steps: attachment and penetration, plus strand synthesis, minus strand synthesis and packaging, maturation and release.

1.2.2.1. Attachment and penetration

The attachment and subsequent penetration of RV is a complicated process. Rotavirus VP4 spikes can interact with several cellular receptors including terminal or sub-terminal sialic acid (Dormitzer et al., 2002a, 2002b), internal sialic acid (Haselhorst et al., 2009; Strains, 1999) and histo-blood group antigens (Hu et al., 2012a; Huang et al., 2012; Ramani et al., 2013) (**Figure 1. 2a**). The interaction with sialic acid has been shown to depend on the genotype of genome segment 4 encoding VP4 and attachment is mediated by VP8* (Dormitzer et al., 2002a, 2002b). Various molecules on the surface of cells (Desselberger, 2014) such as, integrins (α2β1, αvβ3, αxβ2, α4β1) (Coulson et al., 1997; Graham et al., 2003; Gutiérrez et al., 2010; Zarate et al., 2004) or heat shock protein 70 (Guerrero et al., 2002; Perez-Vargas et al., 2006; Zárate et al., 2003), act as post-attachment receptors which interact with ligand motifs on VP5* or VP7. As RV encounters the above cellular receptors, the VP4 spikes change to a 'post-penetration umbrella' conformation, where VP5* is now exposed on the surface (Kim et al., 2010; Settembre et al., 2011; Trask et al., 2010). The precise mechanism of cell penetration remains unknown and it may occur by receptor-mediated endocytosis or direct membrane penetration (Ludert et al., 1987) (**Figure 1. 2b**). The low Ca^{2+} concentration within endosomes lead to the solubilisation of the outer capsid proteins to yield DLPs (Cohen et al., 1979; Ludert et al., 1987).

1.2.2.2. Plus strand synthesis

Protein and genome synthesis in rotavirus are directed by plus strand RNAs (**Figure 1. 2c**). The transcription complexes (TC) within DLPs (Aponte et al., 1996) are tethered to RNA segments and are thus responsible for transcription of the specific genome segment (Periz et al., 2013). After the uncoating of TLPs, DLPs use minus strand genomic RNA to produce non-polyadenylated, (+) ssRNA, which are subsequently released through the channels into the

cytoplasm (Silvestri et al., 2004). Large amounts of mRNA are continuously produced by transcriptionally active DLPs as long as sufficient precursors and ATP are provided (Cohen et al., 1979; Lawton et al., 1997; Lu et al., 2008; Spencer and Arias, 1981). Furthermore, the production of mRNA logarithmically increases when newly synthesized DLPs become transcriptionally active (secondary transcription) (Stacy-Phipps and Patton, 1987). After the release of segment-specific (+) ssRNA from DLPs, translation of proteins occurs in the cytoplasm.

1.2.2.3.   Minus strand synthesis and packaging

Minus strand synthesis is signalled by a conserved sequence at the 3' end of RV mRNA (Chen and Patton, 1998). The initiation of mRNA synthesis by RNA polymerase on the dsRNA genome segments remains unknown. VP2 plays a crucial role in the packaging and activation of VP1 (Boudreaux et al., 2013). VP1 and VP3 interact with the 11 (+) ssRNA and are packaged within VP2 to form single layered particles (SLPs) (Trask et al., 2012). During the packaging process, divalent cations or trivalent cationic compounds are packaged along with the negatively charged RNA to neutralise the RNA (Desselberger et al., 2013; Gouet et al., 1999) (**Figure 1. 2d**). Both the molecular mechanism of the formation of SLPs and the packaging of genome segments within individual SLPs remain unknown. The addition of VP6 to the SLPs results in the formation of DLPs (Desselberger et al., 2013; Trask and Dormitzer, 2006). Minus strand replication and assembly of the DLP occur in viroplasms.

1.2.2.4.   Maturation and release

Double-layered particles formed in viroplasms bud into the ER in a process that involves NSP4 as a intracellular receptor which interacts with VP6 of DLPs (Taylor et al., 1996). A DLP-VP4-NSP4 complex buds into the ER after several successive interactions of DLPs with NSP4 tetramers (Trask et al., 2012). After budding into the ER, VP4 is secured into position when the transient membrane is replaced by VP7. The origin, function and mechanism by which the transient membrane is removed requires further research (Desselberger, 2014). It is postulated that VP6 interacts with trimers of VP4 first and subsequently embeds into VP7 (Trask and Dormitzer, 2006). The addition of VP7 to form TLPs finalizes the assembly of the RV virion (**Figure 1. 2e**). How RV virions are released from cells depend on the polarisation (**Figure 1. 2f**) (Trask et al., 2012) of cells as virions exit non-polarised by direct lysis (Musalem and Espejo, 1985), while they exit polarised cells by trafficking and secretion from the apical cell surface (Jourdan et al., 1997).
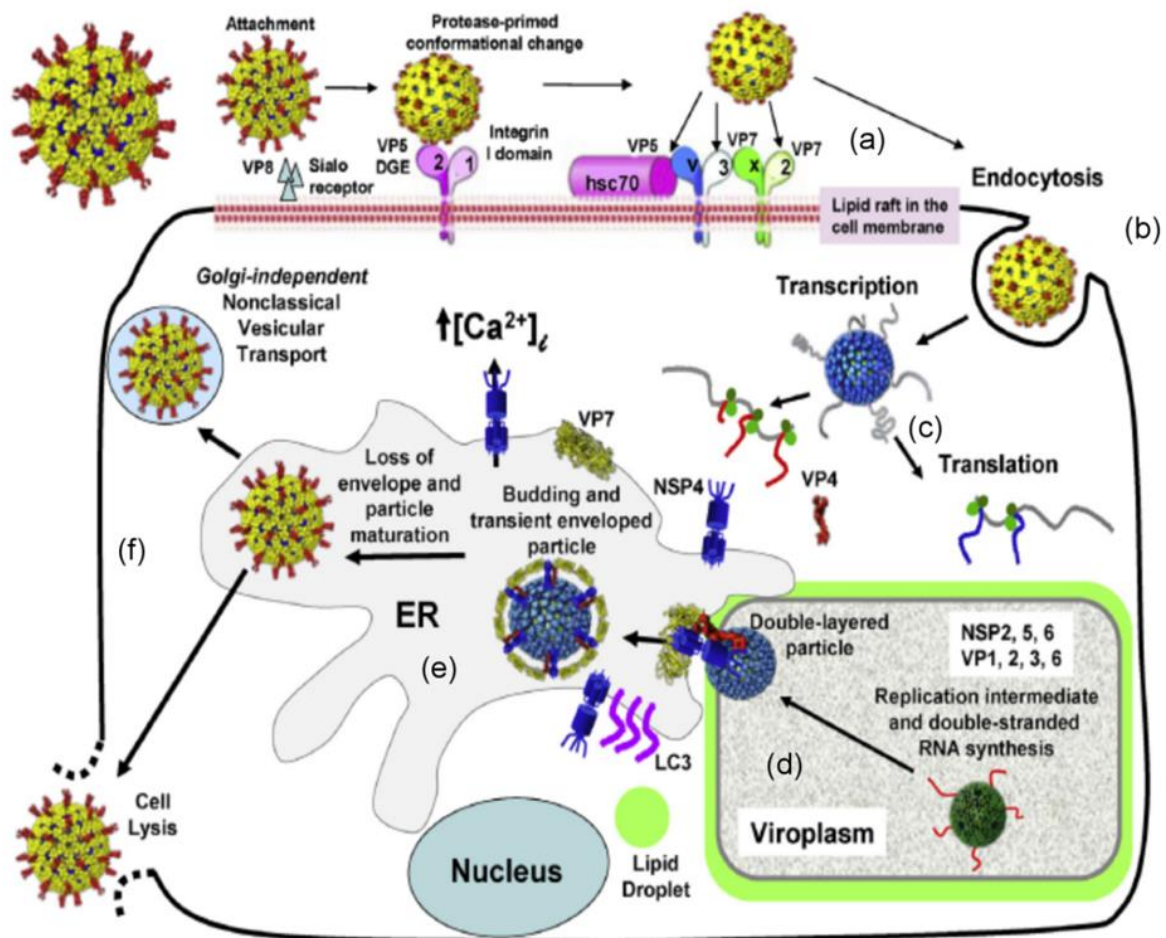
Figure 1. 2. An overview of the replication cycle of rotavirus. (a) Rotavirus VP8* attaches to the target cell. (b) VP5* mediates penetration of rotavirus by different mechanisms, that includes endocytosis. (c) Transcription of (+) RNAs occurs when the triple-layered particle loses its outer capsid and double-layered particles activate the internal polymerase complex. (d) The formation of viroplasms, by NSP2/VP2 and NSP5 interacting, and along with VP1, 3 and 6, leads to viral replication and packaging. (e) The current model of assembly proposes a complex of NSP4, DLPs and VP4. The complex buds into the ER via an undefined mechanism. After budding into the ER, NSP4 allows the assembly of VP7 and the formation of the TLP. (f) Release exposes VP4 to trypsin-like proteases which cleave VP4 into VP5* and VP8* to produce the fully infectious virion. Modified from Desselberger (2014). <mark>Permission obtained from publisher.</mark>

1.3. Viroplasms

1.3.1. General

During RV replication insoluble inclusion bodies, viroplasms, are found in the cytoplasm when viral RNAs and several RV proteins interact (Novoa et al., 2005). These viroplasms consist of viral proteins functioning as viral factories that include VP1-3, VP6, NSP2 and NSP5 (Eichwald et al., 2004) (Patton et al., 2006). Several viruses, such as cauliflower mosaic virus (Bak et al., 2013) and vaccinia virus (Sodeik et al., 1993), utilize viroplasms during their replication cycles. During viral infection several viroplasms can form within a single cell and appear dense

under electron microscopy when stained with uranyl acetate in 50 % ethanol (Altenburg et al., 1980). The successful formation of viroplasms depends on the interaction of a function cellular proteasome and viral proteins, as the inhibition of proteasomal activity effects the level of viral proteins, RNA and virion yield (Contin et al., 2011).

1.3.2. Formation

Viroplasms form 2 - 3 h after the production of viral mRNA commences (Eichwald et al., 2004). Lipid droplets are used by NSP2 and NSP5 to form viroplasm-like structures (VLS). After formation of the VLS, (+) ssRNA and several other viral proteins that include VP1-VP3 and VP6 are recruited to the VLS (Cheung et al., 2010; Fabbretti et al., 1999). Viroplasm-like structures can occur between NSP5 and either NSP2 or VP2, but in the absence of NSP5, no VLSs form, demonstrating that NSP5 plays a central role in VLS assembly (Contin et al., 2010). The C- and N- terminals of NSP5 is crucial for interactions with NSP2 (Eichwald et al., 2004). As discussed earlier, NSP5 can be phosphorylated by both VP2 and NSP2, but this phosphorylation has no effect on the formation of VLS (Afrikanova et al., 1998; Contin et al., 2010). Structurally, NSP5 has been found more concentrated in external regions of viroplasms (Eichwald et al., 2004; Petrie et al., 1984).

1.3.3. Function

Due to the inherent high affinities of RV structural proteins for each other, virus-like particles (VLPs) without any viral RNA can form readily when no viroplasms are present (Patton et al., 2006). Viroplasms ensure that the assembly of the capsid proteins, genome packaging and replication is highly coordinated. In addition, viroplasms also facilitate the recruitment of inner capsid proteins and regulate their spatial and temporal interactions. Although transcriptionally-active DLPs are known to occur near viroplasms (Patton et al., 2006), there is still some debate on whether the (+) ssRNA is made in the viroplasms or transported from the cytoplasm into the viroplasm (Silvestri et al., 2004). Furthermore, viroplasms provide protection from the innate antiviral responses (Patton et al., 2006). Viroplasms are also known to change the microtubule (MT) network within infected cells (Eichwald et al., 2012; Martin et al., 2010). NSP2 binds to the MT network via its positively charged grooves which induces the collapse and rearrangement of the MT network within infected cells (Martin et al., 2010). It was suggested that NSP2 is involved in viroplasm nucleation at tubulin granules and sequestering tubulin in viroplasms. This leads to the depolymerisation of the MT network and inhibits cellular trafficking and functions. Eichwald and co-workers (2012) showed that the MT network is essential in the fusion and condensation of the viroplasm to the perinuclear area of the cell.

1.4. Lipid droplets

1.4.1. Structure

Lipid droplets (LDs) have a phospholipid monolayer exterior and an interior that consist of neutral lipids (triacylglycerols and sterol esters) (Martin and Parton, 2006). This structure allows for the separation of the organic and aqueous phase of the cell. Several different proteins including structural proteins, lipid-synthesis enzymes, lipases and membrane-trafficking proteins have been found in LDs (Brown, 2001; Cohen, 2018; Ducharme and Bickel, 2008; Kuerschner et al., 2008). PAT family, [perilipin (**P**LIN), adipose differentiation-related protein (**A**DRP) and tail-interacting protein (**T**IP)-47 (Miura et al., 2002)] proteins, constitute the majority of proteins found in LDs. The presence of proteins associated with and spanning the membrane along with ribosomal structures, ribosomal associated proteins and RNA interacting proteins have also been found in LDs (Bozza and Viola, 2010). Proteins with predicted membrane insertion such as caveolin and cyclooxygenase (COX) have also been seen localizing with lipid droplets via microscopy (Bozza et al., 1997; Dvorak et al., 1992; Robenek et al., 2005). Adding to the complexity of LDs, they can contain several different types of proteins and can acquire triacylglycerols at different rates (Ducharme and Bickel, 2008; Kuerschner et al., 2008). Thus, a single cell could contain several distinct and unique LDs (Guo et al., 2009). It is believed that proteins embed themselves into monolayers of LDs by using long membrane-embed domains (Martin and Parton, 2006) or by making use of amphipathic helixes to enter the monolayers (Guo et al., 2009). Proteins have been found within the hydrophobic core of LDs, but how they are transported in and out of the hydrophobic core is not clear (Robenek et al., 2005). These observations are suggestive of sub-compartments within the lipid droplet when lipid droplets are formed from ER-derived membranes.

1.4.2. Formation

The formation of LDs commences after FA (carried by albumin and lipoproteins) enter the cell or after internal FA are metabolized (Guo et al., 2009). While some FAs can be produced during the synthesis of carbohydrates (Guo et al., 2009), other FAs enter cells via passive diffusion, by transport proteins or translocases (Ehehalt et al., 2006). Diaglycerols are generated by glycerolipid-synthesis enzymes, which use fatty acyl-CoA (FAs conjugated with coenzyme A (CoA)) as a substrate. Diaglycerols are subsequently converted to either neutral lipids (triacylglycerols) or enter the phospholipid pathway. How the flux between neutral lipids or phospholipids is regulated, remains uncertain. The sterols required for LD formation come from the lysosomal degradation of lipoproteins or are absorbed via endocytosis. The mechanism behind the accumulation of neutral lipids and sterols in LDs are mostly unknown.

However, three models have been proposed for LD formation. The ER domain and budding model (**Figure 1. 3a**) suggests that neutral lipids accumulate between the bilayers of the ER and then bud into the cytoplasm taking phospholipids from the cytosolic membrane. The bicelle model (**Figure 1. 3b**) suggests that neutral lipids accumulate between the leaflets of the ER membrane and LDs are excised by the formation of a bicelle from the membrane taking phospholipids from both the cytosolic and luminal leaflets (Ploegh, 2007). The vesicular budding model (**Figure 1. 3c**) suggested that small bilayers vesicles are used as a platform for making LDs (Walther and Farese, 2009). The neutral lipids are pumped into the vesicle bilayer, eventually squeezing the vesicular lumen until it becomes a small inclusion with the LDs. Recently, experimental evidence was obtained to support the ER domain and budding model with fat-storage and-inducing transmembrane (FIT) proteins playing a facilitating role (Choudhary et al., 2016).

Based on the size and lifecycle stage of LDs, they can be divided into two types, initial LDs (iLDs) and expanding LDs (eLDs) (Kory et al., 2016). The iLDs are formed from the ER, as previously mentioned, and range from 300 to 600 nm in diameter. They bud and detach from the ER in mammals, but remain attached to the bilayer in yeast (Kassan et al., 2013; Wilfling et al., 2013). Expanding LDs are produced from a subset of iLDs with distinct proteins, with the Afr1/COPI machinery required for this transition (Wilfling et al., 2013). Choudhary and co-workers (2016) also found that FIT proteins are essential for the promotion of proper budding from the ER. The exact mechanism of this control still needs to be elucidated. Lipid droplets can range in size from 40 nm to 100 μm and various models for the growth in size have been proposed in which LDs might remain attached to the ER (Guo et al., 2009) and fusion of LDs (Boström et al., 2007). It has been shown that the family of phospholipase A_2 (PLA_2) (enzymes responsible for the hydrolysis of the FA present at the sn-2 position of phospholipids) are responsible for the regulation of various steps in LD biogenesis. This includes providing free FA from membrane phospholipids for neutral lipid synthesis (Akiba et al., 2003; Gubern et al., 2009b; Leiguez et al., 2011), modifying phospholipid-containing particles to facilitate their internalization by cells (Boyanovsky et al., 2005; Hanasaki et al., 2002), generating metabolites that control LD formation (Pucer et al., 2013) and being directly involved in the formation process (Gao et al., 2017; Gubern et al., 2008, 2009a; Guijas et al., 2012).
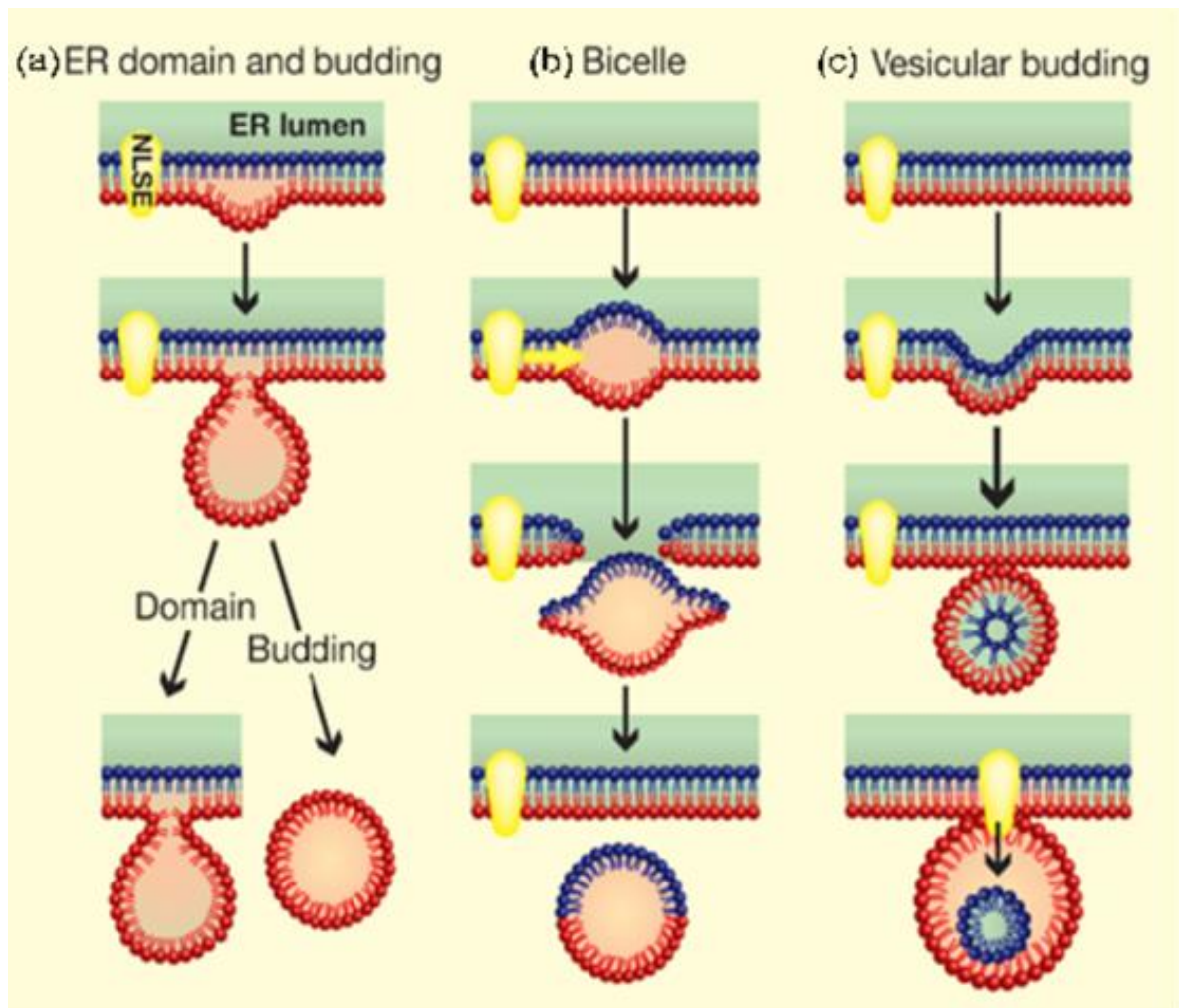
Figure 1. 3. The proposed models for the formation of lipid droplets.

(a) ER domain and budding model. (b) Bicelle model. (c) Vesicular budding model (Guo et al., 2009). <mark>Permission obtained from publisher.</mark>

1.4.3. Lipids

Lipids are defined as small hydrophobic or amphiphilic molecules allowing them to form structures such as membranes, vesicles and liposomes (Fahy et al., 2009). Lipids originate completely or in part from keto-acyl and isoprene; two distinctive types of biochemical subunits. Lipids are divided into eight categories, dependent on the two biochemical subunits (ketoacyl and isoprene groups): FA, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides, sterol lipids and prenol lipids (Fahy et al., 2011). The main biological functions of lipids include storing energy, signalling, and acting as structural components of cell membranes (Subramaniam et al., 2011). As previously mentioned, LDs consist of neutral lipids and phospholipids (Martin and Parton, 2006).

1.4.3.1. Neutral lipids

The neutral lipids within LDs are predominantly triacylglycerols (TAGs) or cholesteryl esters (Guo et al., 2009). Neutral lipids are nonpolar, water-insoluble substances consisting of FA and glycerol (Voet and Voet, 2011). D'Aquila and co-workers (2015) showed that when mice are administered an olive oil bolus, the neutral lipids accumulate in distinct regions within enterocytes from the small intestine, namely LDs. Cholesteryl ester, a derivate of cholesterol, along with the glycerophospholipids and sphingomyelins are important components of membrane lipids (Bach and Wachtel, 2003). Cholesteryl esters have a lower solubility in water due to their increased hydrophobicity. Triacylglycerols are esters derived from glycerol and three FAs. They are the most abundant class of lipids in mammalians as they are the major source of energy although they do not form part of biological membranes (**Figure 1. 4a**) (Voet and Voet, 2011). Fatty acids are made up of a hydrocarbon chain containing a polar and non-polar end within a diverse group of molecules (Vance and Vance, 2015). Typically, the carbon chain can be between four and 24 carbons long and may be saturated or unsaturated. The carbon chain may contain functional groups such as oxygen, halogens, nitrogen and sulphur. The synthesis of FA from acetyl-CoA and malonyl-CoA involves seven enzymatic reactions that yield mainly palmitic acid which is the precursor of longer chain saturated and unsaturated fatty acids through the actions of elongases and desaturases (**Figure 1. 4b**) (Voet and Voet, 2011). Saturated FA contain no double bonds, while unsaturated FA can contain one (mono-) or more (poly-) double bonds. Unsaturated FA can either be *cis* (two hydrogen atoms adjacent to the double bond stick out on the same side of the chain) or *trans* (two adjacent hydrogen atoms lie on opposite sides of the chain) FA. *Cis* bonds cause FA to bend, and occur more naturally than *trans* bonds (Voet and Voet, 2011). Unsaturated FA are mainly incorporated into the phospholipids of membranes (Carrillo et al., 2012). These FA can affect cellular function directly or indirectly. There are several hypotheses for how these mechanisms work including: increase in signal transduction, decreasing arachidonic acid (AA) content, modulation of intracellular pathways and modulation of gene expression. Examples of biologically important FAs include the eicosanoids, derived primarily from AA and eicosapentaenoic acid which in turn comes from linoleic acid and α-linolenic acid, respectively (Dennis and Norris, 2015).

Polyunsaturated FA can be subdivided into omega-3 (ω-3) or omega-6 (ω-6) FAs depending on which carbon from the methyl end, the last double bond occurs (Scorletti and Byrne, 2013). Omega-3 and omega-6 are both essential for cell membranes and act as precursors for various other substances in the host (Simopoulos, 2002). Omega-3-fatty-acid-derived signalling molecules are anti-inflammatory while omega-6-fatty-acid-derived signalling

molecules are pro-inflammatory. Omega-3 FA lower the risk of cardiovascular disease and lower the inflammatory responses (Calder, 2010) while the opposite is true for omega-6 FA (Innes and Calder, 2018). Husson and co-workers (2016) showed that supplementation with ω-3 FAs can have both a beneficial and deleterious effect on the prevention and control of infectious diseases. They showed that in some instances (0.5 g/day of ω-3 FA) ω-3 FA supplementation can induce a strong anti-inflammatory response by switching from pro-inflammatory PGE_2 and leukotriene-4 towards less inflammatory products such as PGE_3 and LTX5 and inhibition of NF-κB via signalling pathways and peroxisome proliferator-activated receptors activation. In other instances, (2-4-fold higher dose/day of ω-3 FA) ω-3 FA supplementation can lead to changes in the gut microbiota composition by increasing anti-inflammatory bacterial species, which reduce local and systematic inflammation and impair immunity.
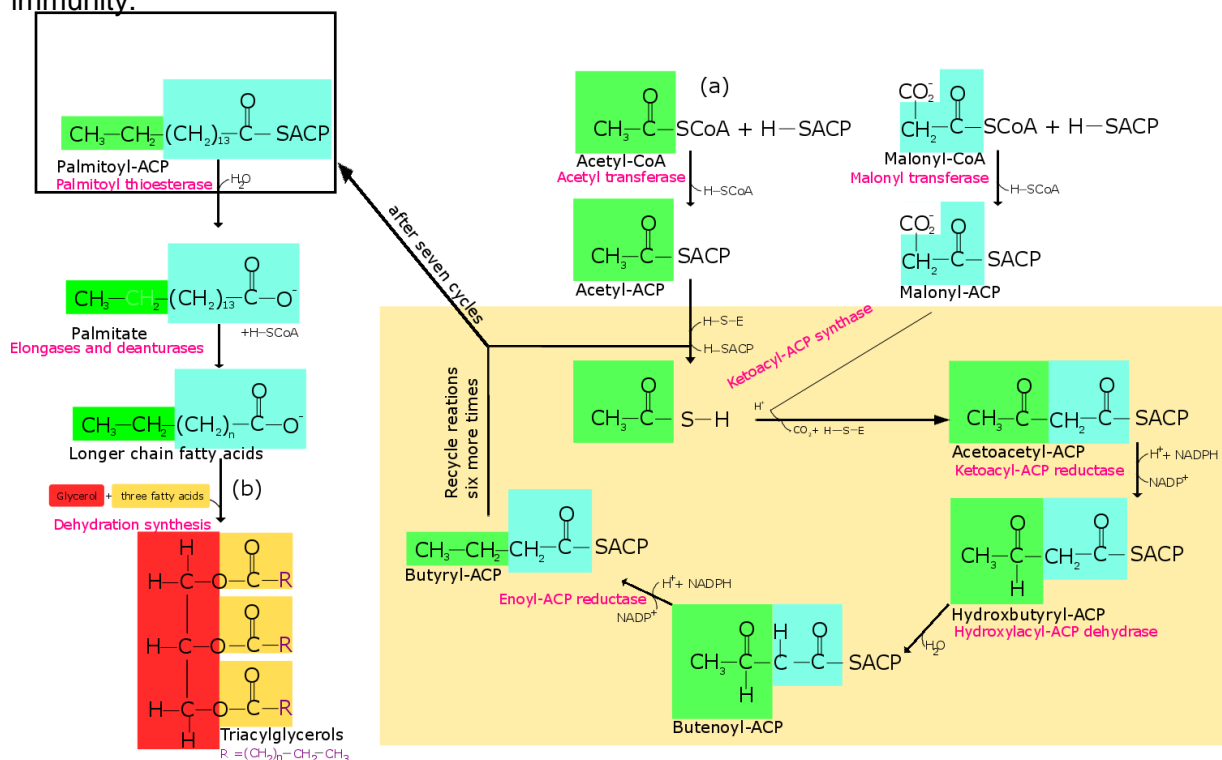
Figure 1. 4. Reactions for the biosynthesis of fatty acids and triacylglycerols. a) Seven cycles of C2 elongation are required to form palmitate which is the precursor to triacylglycerols. b) Biosynthesis of triacylglycerols occurs when glycerol and three fatty acids (any combination of fatty acids) react in a dehydration reaction. Compiled from Voet and Voet (2011).

1.4.3.2. Phospholipids

Phospholipids form the monolayer around LDs (Guo et al., 2009). Phosphatidylcholine (PC) is the most prevalent phospholipid constituting 60 % of the monolayer (Chitraju et al., 2012). Phosphatidylcholine is followed by 24 % of phosphatidylethanolamine (PE) and 8 % of phosphatidylinositol (PI). Phosphatidylinositol, as a signalling lipid, has been implicated in viral

infection (Helms et al., 2015). Phosphatidic acid (PA) is not found in significant amounts within LDs, while its downstream product diacylglycerol (DG) can accumulate on LDs in significant amounts, but its distribution between the core and surface of lipid droplets is unknown (Penno et al., 2013). Treatments that either generate or promote the accumulation of DG have been shown to recruit exchange-able lipid-binding proteins (proteins that are stable when not bound to lipid), but the functional implications are unknown (Skinner et al., 2009). Phosphatidylcholine, PE and PI are all synthesised in some part from DG (Voet and Voet, 2011). Both the active phosphate esters of the polar head groups of choline and ethanolamine react with the C3 OH group of DG to form a phosphodiester bond forming PC and PE, respectively (**Figure 1. 5a**). The liver can also convert PE to PC by trimethylating the amino group via S-adenosylmethionine. In inositol the hydrophobic tail is activated which then reacts with the C1 OH group to form a phosphodiester bond forming PI (**Figure 1. 5b**). Cells grown in the absence of choline accumulated more numerous and larger lipid droplets (Brown et al., 2016). Interestingly the deficiency in choline also changes the lipid droplet binding of a subset of proteins. Phosphatidylcholine has been shown to play a critical role in the maintenance of LD stability (Krahmer et al., 2011). The most obvious function of the phospholipids is the formation of a boundary that separates the hydrophobic core of the lipid droplet with the aqueous environment (Penno et al., 2013). Other than the separation function, the lipid monolayer could also be responsible for the differential recruitment of lipid droplet proteins.
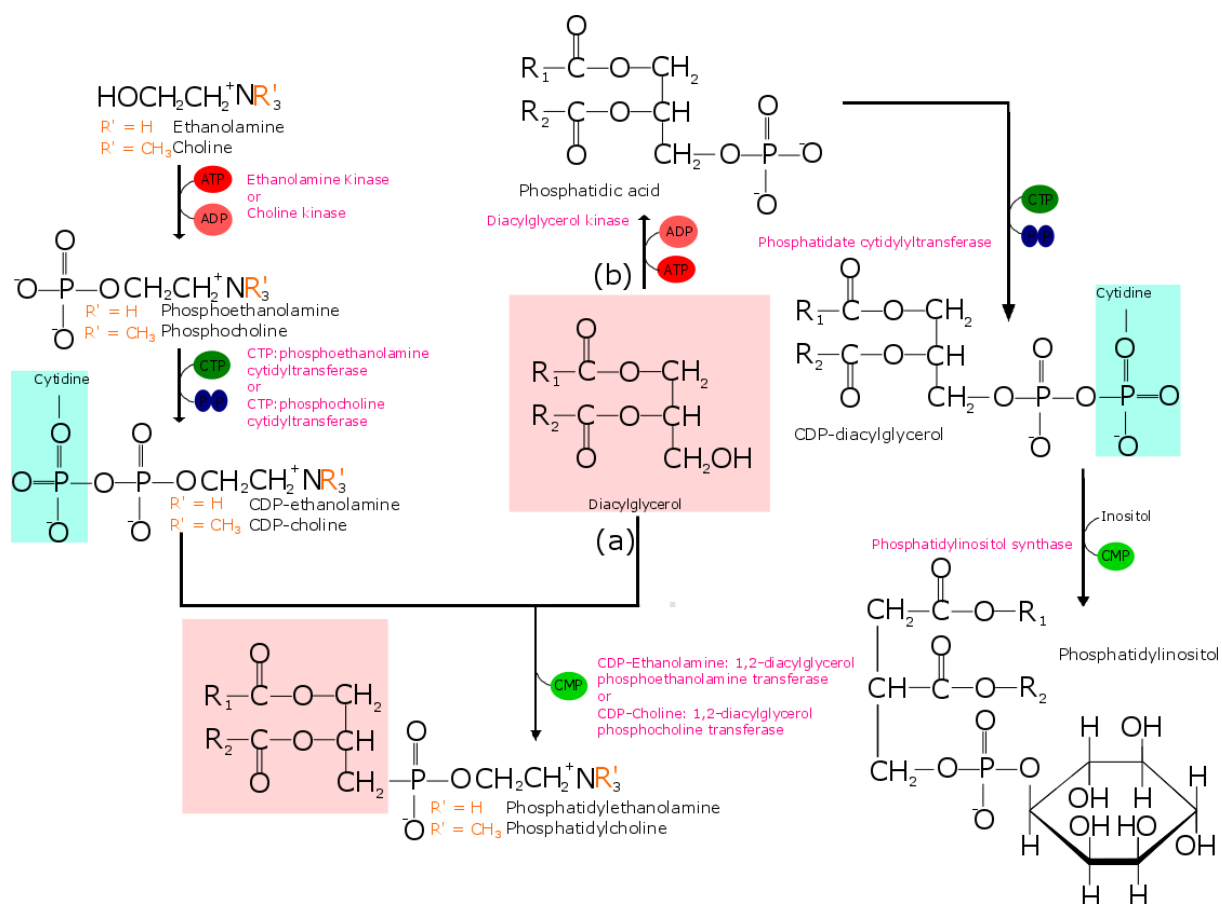
Figure 1. 5. The synthesis pathways of phospholipids. Diacylglycerol is the major precursor to phosphatidylethanolamine (PE), phosphatidylcholines (PC) and phosphatidylinositol (PI). (a) Ethanolamine or choline are activated by ATP and then by CTP. The subsequent CDP-ethanolamine or CDP-choline then reacts with the C3 OH group of DG to form phosphodiester bonds leading to PE and PI. Phosphoethanolamine can also be converted to PC within the liver (not shown). (b) Diacylglycerol is converted to phosphatidic acid and then to CDP-diacylglycerol which reacts with inositol to form PI. Compiled from Voet and Voet (2011).

1.4.3.3. Sphingolipids

Sphingolipids are a class of lipids that have regulatory, structural and metabolic functions (Deevska and Nikolova-Karakashian, 2017). Sphingolipids are chemically distinct from neutral lipids and phospholipids (they have no glycerol backbone or ester/ether- linked chains), but have nonetheless been found in LDs (Loizides-Mangold et al., 2014). Most studies performed to elucidate the functions of sphingolipids, have delineated ceramide as the key bioactive sphingolipid (Deevska and Nikolova-Karakashian, 2017). These studies have found that sphingolipids play a role in the formation and size of LDs (Holopainen et al., 2000), the rate of lipogenesis and lipolysis (Aguilera-Romero et al., 2014), the degradation of LDs via autophagy (Zheng et al., 2006), LD cholesterol content (Leventhal et al., 2001) and binding of LD proteins (Krönke, 1999). Although these functions are known, more research is needed to elucidate

the molecular mechanisms behind them and ongoing research on sphingolipid metabolic and signalling networks may yet reveal a clearer picture of their role in LD biogenesis (Deevska and Nikolova-Karakashian, 2017).

1.4.3.4. Eicosanoids

Eicosanoids, derived from ω-3 or ω-6 FA, are signalling molecules that exert complex control over many biological systems (Decaterina, 2001). Eicosanoids are primarily generated through an oxidative pathway from AA (Harizi et al., 2008), although some eicosanoids can be produced from ω-3 polyunsaturated FAs (Calder, 2005). Lipid droplets within host cells facilitate the production of lipid immunomodulators, mainly eicosanoids (Meester et al., 2011). Lipid droplets have been shown to contain stores of AA, showing that LDs can initiate cascades that form eicosanoids (Weller et al., 1991).

Eicosanoids are produced from the oxygenation of FA by three families of enzymes, cyclooxygenase (produces the prostanoids from AA), lipoxygenase (produces the leukotrienes from AA) and epoxygenases (numerous cytochrome P450 enzymes (which metabolizes toxic compounds) (**Figure 1. 6**). Eicosanoids are not stored within cells and their biosynthesis is only activated by mechanical trauma, cytokines, growth factors or other stimuli (Funk, 2001; Soberman and Christmas, 2003).
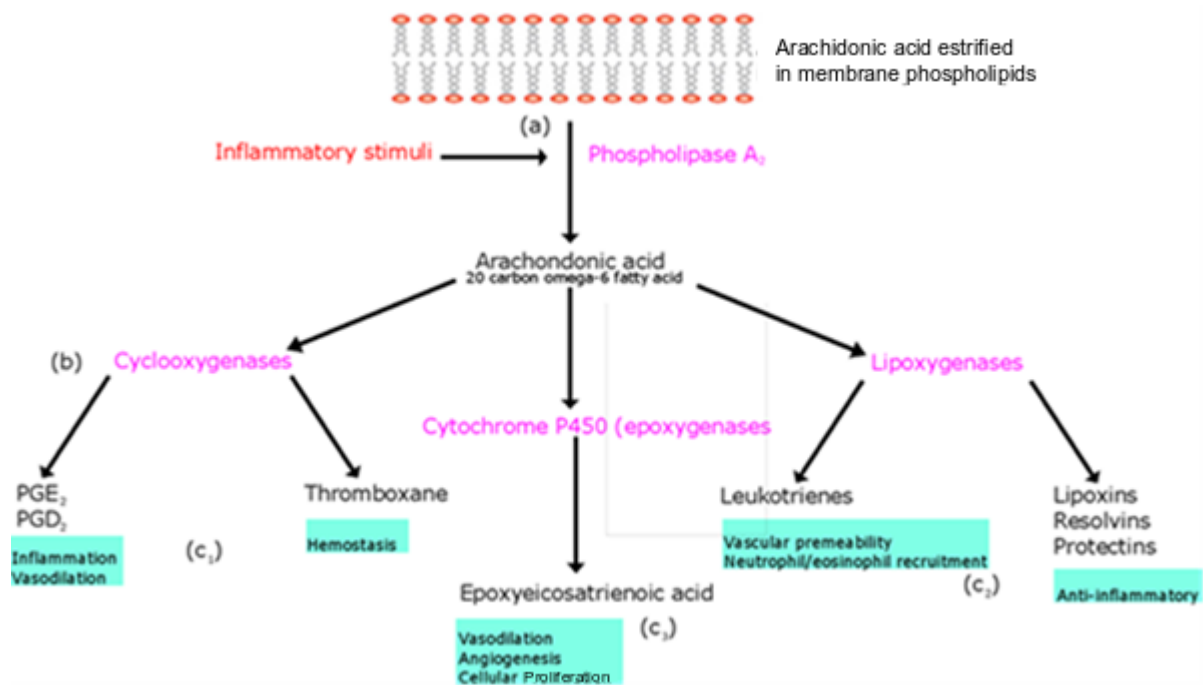
Figure 1. 6. The eicosanoid synthesis pathway from AA. There are parallel pathways for eicosapentaenoic acid and dihomo-γ-linolenic acid which are not shown. a) Inflammatory stimuli releases phospholipases which frees AA from phospholipids or diacylglycerol. b). Arachidonic enters one of several pathways to produce various eicosanoids. c1) Effects

prostanoids have on their targets. c2) Effects leukotrienes have on their targets. c3) Effects eoxins have on their targets. Modified from Pratt and Brown (2014). <mark>Permission obtained from publisher.</mark>

Prostanoids mediate vasoconstriction/vasodilation, coagulation, pain and fever (Funk, 2001). Leukotrienes use lipid signalling to convey information to either the cells producing them (autocrine signalling) or neighbouring cells (paracrine signalling) in order to regulate immune responses (Dahlén et al., 1981). Epoxygenases have vasodilating actions on the heart, kidney and other blood vessels. They may also act to reduce inflammation, promote the growth and metastasis of certain tumours, promote the growth of new blood vessels, regulate the release of neuropeptide hormones in the central nervous system, and in the peripheral nervous system they inhibit or reduce pain perception (Spector and Kim, 2015). Leukotrines have anti-inflammatory effects (Aliberti, 2005) and have the ability to diminish the production of interleukin 12 (cytokine) and consequently the cellular immune response (Aliberti et al., 2002). Eicosanoids function by binding to membrane receptors (Harizi et al., 2008). This binding can result in an increase or decrease in the production of cytosolic second messenger, activation of protein kinase or a change in membrane potential. All the enzymes responsible for the release of AA from glycerophospholipids (**Figure 1. 7a & b**) and those involved in the conversion of AA into eicosanoids (**Figure 1. 7c & d**) were shown to co-localize within LDs (Bozza and Viola, 2010; Wenguiyu et al., 1998).
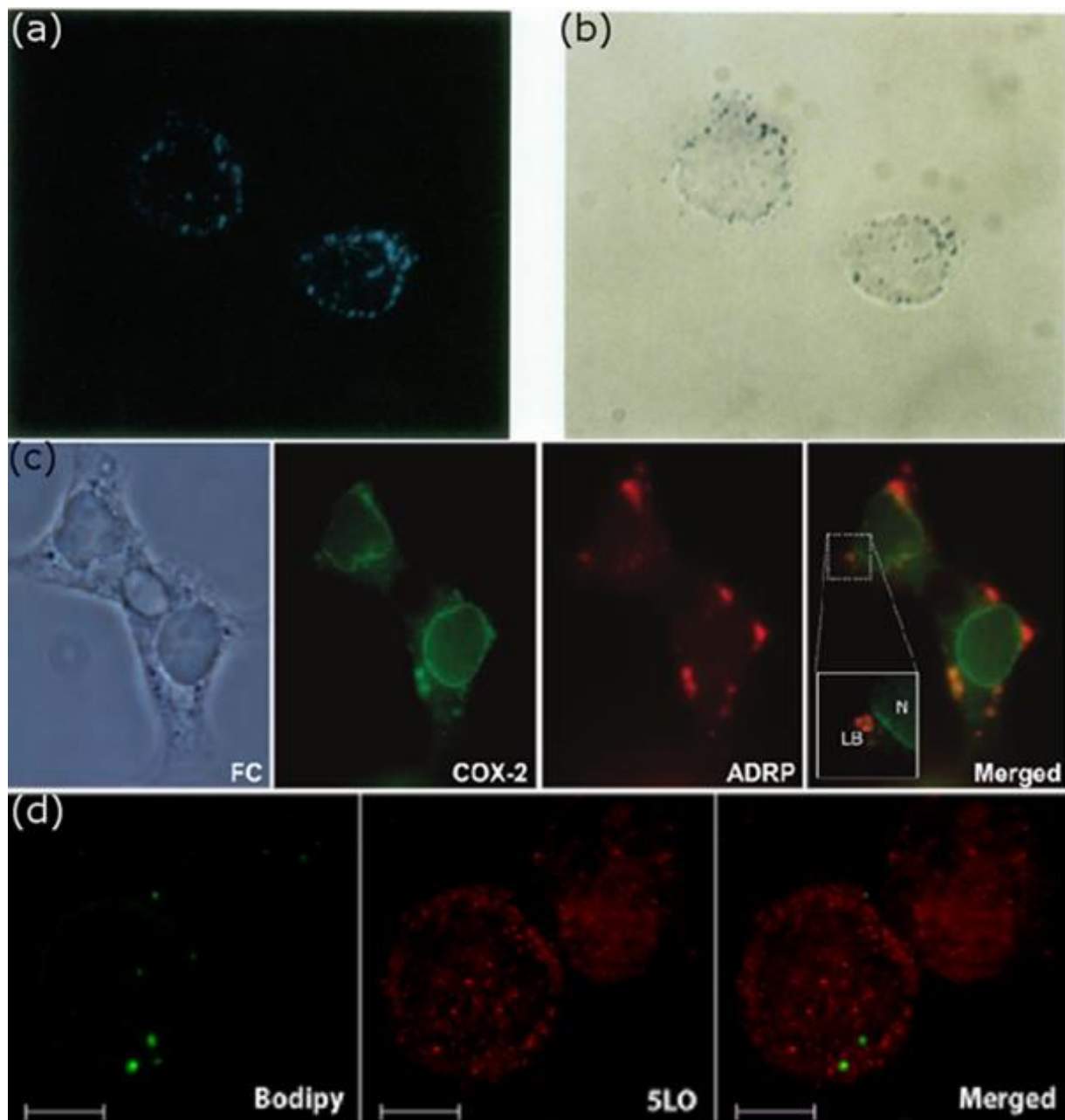
Figure 1. 7. Various staining techniques used to show the co-localizing of lipid droplets and eicosanoid forming enzymes. a) Lipid droplets of U397 cells labelled with fluorescent fatty acid 1-pyrenedodecanoic acid (b) and the corresponding cells labelled with anti- calcium-dependent phospholipase A_2 which releases AA from glycerophospholipids (Wenguiyu et al., 1998). c) Lipid droplets of CACO-2 cells under phase contrast followed by staining with ADRP and anti-COX-2 (Accioly et al., 2008a). d) Lipid droplets of macrophages stained with BODIPY and anti-5-LO (primary anti-body against 5-leukotriene) (Silva et al., 2009). <mark>Permission obtained from publisher.</mark>

1.4.4. Proteins

The proteins of LDs are some of its best understood components of LDs (Martin and Parton, 2006; Yang et al., 2012). Lipid droplet-associated proteins vary between cell and tissue type and can number in the hundreds (Kory et al., 2016). Seipin, a homo-oligomeric integral

membrane protein in the ER, concentrates at junctions with LDs (Cartwright et al., 2015). Additionally, seipin has been identified as a key regulator of LD biogenesis and homogenous morphology and content. Seipin also regulates the metabolism of PA at the LD-ER contact, acting as a scaffold to recruit PA-metabolism enzymes (Sim et al., 2013; Talukder et al., 2015). Multiple observations have shown that LDs deficient in seipin are at least partly dysfunctional (Chen et al., 2012; Wang et al., 2014; Wolinski et al., 2011). Perilipin (PLIN) is the best characterized LD-associated protein and has key functions in stabilizing the storage of neutral lipids (Itabe et al., 2017). Perilipin is characterized by a PAT domain, where PAT refers to a region of sequence similarity that is also present in other proteins such as **P**LIN (PLIN-1), **A**DRP (PLIN-2) and mannose-6-phosphate receptor binding protein (**TI**P-47) (PLIN-3). These proteins are more commonly known as perilipin-1 (A), -2 and -3. Although the exact molecular mechanism of PLIN at the surface of LDs is yet to be elucidated, it directly participates in the activation of lipolysis and functionally interacts with hormone-sensitive lipase (HSL) (Sztalryd et al., 2003). Lipolysis occurs when cyclic-AMP-dependent protein kinase A (PKA) mediates the phosphorylation of both PLIN and hormone-sensitive lipase (Londos et al., 2005). Hormone-sensitive lipase translocates to the surface where it mediates the hydrolysis of triacylglycerols and sterols (Moore et al., 2005). The phosphorylation of PLIN is essential for the translocation of HSL to the surface (Sztalryd et al., 2003). Significantly less is known about the activities of the other members of the PAT-family, although they probably function in the same manner (Martin and Parton, 2006).

Adipose differentiation-related protein binds FA (Serrero et al., 2000) and cholesterol (Atshaves et al., 2001) promotes the accumulation of triacylglycerols and stimulates fatty acid uptake. Adipose differentiation-related protein directly binds to ARF1 which activates phospholipase 1 (PLD1) (Martin and Parton, 2006). ARF1 and activated PLD1 both localize to LDs. Phospholipase 1 is involved in the regulation of diverse biological processes including vesicle trafficking, membrane fusion and cytoskeletal reorganization. In addition, several Rab proteins have been detected within LD fractions (Brasaemle et al., 2004), and they function mainly in membrane-trafficking steps associated with the endosomal system or biosynthetic pathway (Zerial and McBride, 2001). A limited number of LD-associated Rabs have been examined in detail, but they show the possibility of facilitating links between LDs and other organelles or in coordinating LD motility (Martin et al., 2005). Rab18 is of interest as it localizes with active LDs and may mediate increased association with the ER to facilitate the transfer of FA or neutral lipids between the LD monolayer and the bilayer of the ER. Caveolins, integral membrane proteins (Parton, 1996), have also been found to have a regulated localization to LDs (Fujimoto et al., 2001). They cause an increase in intracellular neutral lipid accumulation

and bind to FA and cholesterol (Martin and Parton, 2006). The translocation of caveolin from the cell surface to LDs in response to lipid stimulation is yet to be elucidated.

Work by Ueno and co-workers (2016) in yeast, demonstrated that lipid droplet in sporulation proteins may have a possible role in the maintenance of lipid homeostasis and also in the membrane protein transport. Work done by Gao and co-workers (2017) proved that the cell death-inducing DFF45-like effector protein family is crucial in the regulation of LD fusion and growth. Lipid metabolism enzymes have also been frequently found to associate with LDs. These include: acyl-CoA synthetases, acyltransferase, lipoprotein lipase (Goodman, 2008). Other enzymes associated with LDs are those responsible for eicosanoid production (Bozza and Viola, 2010). Studies have shown that viperin (an ER-associated and interferon-inducible virus inhibitory protein) localises to the surface of the ER and is also particularly abundant on the surface of LDs (Hinson and Cresswell, 2009).

LD proteins are classified based on their localization to LDs (Kory et al., 2016). Class I proteins often have a membrane-embedded, hydrophobic hairpin motif, and access the ER either during formation (Zehmer et al., 2008) or by ER-LD membrane bridges after formation (Jacquier et al., 2011; Wilfling et al., 2013). Class II proteins are translated in the cytosol and bind directly to the surface of LDs by amphipathic helices or via multiple amphipathic and hydrophobic helices (Hristova et al., 1999; Seelig, 2004; Terzi et al., 1997). There are some proteins which do not fall within this classification and it is uncertain how they localize to LDs (Kory et al., 2016). They require lipid modifications and protein-protein interactions. Hung and co-workers (2017) found that two enzymes, acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) and DGAT2 could alter the pools of TAGs involved in the number or size of LDs and the enterocyte LD proteome. They concluded that DGAT1 and DGAT2 function together to regulate the process of dietary fat absorption by preferentially synthesising TAGs for the incorporation into distinct subcellular TAGs in enterocytes. Furthermore, it has been found that monoacylglycerol O-acyltransferase (MGAT1) facilitates TAG synthesis and LD expansion when cells are supplemented with free FAs (Lee and Kim, 2017). They also indicated that MGAT1 and DGAT2 interact with each other, resulting in an increase in TG synthesis and LD expansion.

1.4.5. Function of LDs

Lipid droplets have been implicated in several human pathologies, as they are critical for normal cellular and organismal functions (Cohen, 2018; Krahmer et al., 2013). The role that LDs play in energy homeostasis includes the breakdown of neutral lipids in the core by lipolysis or lipophagy (Zechner et al., 2012). The β-oxidation in the mitochondria and peroxisomes use

the liberated FAs for energy production (Eaton, 2002; Kerner and Hoppel, 2000). Apart from the historical role of LDs their importance in various other biological processes is now coming to light (Ohsaki et al., 2006).

Lipid droplets can store several fat-soluble vitamins and their metabolites. Both vitamin E and vitamin A have been found within LDs, although the exact function of vitamin E storage is still unknown (Traber and Kayden, 1987). The ability of LDs to store vitamin A, allows for a ready store of vitamin A during dietary insufficiency, as vitamin A is esterified to FAs to form retinyl esters (Grumet et al., 2017; Senoo et al., 2007). An accumulation of precursors to steroid hormones and FA signal molecules have also been observed within LDs (Papackova and Cahova, 2015; Shen et al., 2016). When LDs were proteomically analysed, it was found that they co-purify with steroidogenic enzymes, which led to the hypothesis that LDs may function as sites for steriodogenic enzymes and/or the transfer of steroid intermediates (Yamaguchi et al., 2015). As previously mentioned, LDs have also been found to store the precursors of eicosanoids (Bozza et al., 2011).

Endoplasmic reticulum (ER) stress and oxidative stress within the cell can cause severe damage to cellular homeostasis (Ohsaki et al., 2006). Endoplasmic reticulum stress occurs when there is an imbalance between unfolded proteins and their chaperones or an excess of FAs. This imbalance/excess then triggers the unfolded protein response in order to restore protein homeostasis (Oakes and Papa, 2014; Volmer and Ron, 2015) or lipid levels (Volmer et al., 2013). The mitigation of ER stress by LDs is in the storage of triglycerides, with the underlying mechanisms still being investigated (Bosma et al., 2014; Fuchs et al., 2012). By rebalancing lipid homeostasis as well as acting as vehicles to remove misfolded proteins, LDs within the ER can decrease the damage of ER stress (Ploegh, 2007; Vevea et al., 2015). The association of the ER with the LDs is also unique in that highly hydrophobic lipid esters are transported between them (Ohsaki et al., 2017). When reactive oxygen species (ROS) increase to harmful levels that overwhelm cellular defence enzymes, oxidative stress occurs (Ohsaki et al., 2006). Lipid droplets have been shown to protect cancer cells from ROS toxicity during hypoxia by taking up and storing FA (Wang, 2016). In another study it was found that LDs limited ROS toxicity in the nervous system by taking up poly-unsaturated fatty acids (PUFAs) making them less vulnerable to peroxidation chain reactions (Bailey et al., 2015).

Studies have also indicated that LDs may possibly play an active role in the maturation of proteins and the modulation of the proteins folding state (Welte and Gould, 2017). Inclusion bodies (IB), structures rich in chaperones, transiently take up proteins that are destined for refolding (Moldavski et al., 2015). Iml2, a conserved protein found to co-localize with IBs, has been shown to physically interact with known LD proteins, while IBs and LDs are in very close

contact during protein misfolding. Furthermore, it was shown that while the stores of triglycerides had no effect on refolding activity, the lack of sterol esters abolished it. Lipid droplets have also been found to play a role in protein storage (Cermelli et al., 2006; Li et al., 2012). Studies in *Drosophila* have shown that histones co-localize with LDs within the nucleus and this could possibly aid in rapid chromatin assembly, buffer imbalanced histone production or serve as a reservoir of antibacterial agents (Anand et al., 2012; Li et al., 2012, 2014). Interestingly, it has been shown that histones at the surface of LDs can be functional in an intracellular antibacterial defence system (Anand et al., 2012). Further progress in determining if LD histones contribute to embryonic development and innate immunity is hampered due to the lack of identified histone anchors in mammal cells. Other proteins that can be transiently stored in LDs include PLIN5 (Gallardo-Montejano et al., 2016), nuclear factor of activated T cells 5 (Ueno et al., 2013), and the antiviral protein, viperin (Helbig and Beard, 2014). Observations have shown that various proteins accumulate in LDs before they undergo degradation, leading to the proposal that LDs might be required for the turnover of specific proteins (Fujimoto and Ohsaki, 2006b). This in-between step might assist with the degradation of some highly hydrophobic proteins (Fujimoto and Ohsaki, 2006b) or might facilitate the delivery of damaged proteins to the lysosome via autophagy (Ohsaki et al., 2006).

Smaller LDs have been found in the nucleus by several research groups (Layerenza et al., 2013; Uzbekov and Roingeard, 2013), but their physiological function remains to be elucidated. It is speculated that the LDs may modulate transcriptional regulation, control nuclear membrane composition or act as nucleus-specific protein storage sites (Farese and Walther, 2016). Furthermore, LDs undergo active and directed motion (Welte, 2009) and mechanisms driving this motion vary between cells (Welte and Gould, 2017). Lipid droplets have also been shown to be critical for replication and evasion of an immune response towards various pathogens, which will be discussed next.

1.4.6. Role of LDs during infection by pathogens

Various pathogens, including, viruses, bacteria and parasites specifically target host lipid droplets during their life cycle (Roingeard and Melo, 2017). One of the key characteristics of *Mycobacterium tuberculosis* is the formation of 'foamy', LD-filled macrophages (Russell et al., 2010). *M. tuberculosis* moves to the LD and once inside of the LD starts to accumulate its own lipid inclusions and then enters a phase of lipid based metabolism (Munoz-Elias and McKinney, 2005). Lipid droplets are thus a major source of energy and carbon for *M. tuberculosis* during chronic infection (Roingeard and Melo, 2017). Both *M. bovis* (D'Avila et al., 2006) and *M. leprae* (Mattos et al., 2011) infections lead to the accumulation of LDs within host cells. Host-derived fatty acids are imported into the bacterial LDs and used for the

synthesis of triglycerides (Daniel et al., 2011). *Chlamydia trachomatis* is dependent on the lipids of the host to form the reticular body (replicative form). This process requires large amount of phospholipids which are in part recruited from internalized LDs (Cocchiaro et al., 2008). *C. trachomatis* induces the translocation of LDs from the host cytoplasm into the lumen of the parasitophorous vacuole containing the bacterium, in an endocytosis-like process. The capture of host triglycerides and cholesterol esters by *C. trachomatis* appears to be crucial for bacterial growth during propagation. *C. muridarum* has been shown to incorporate LDs into bacterium-containing vacuoles *in vivo*, in a mouse model (Rank et al., 2011).

Several protozoan parasites simulate the formation and accumulation of LDs in both immune and non-immune cells (Toledo et al., 2016). *Trypanosoma cruzi* has been documented to increase the size of LDs as well as cause ultrastructure changes. This pathology allows for a strong inflammatory response related to an influx of macrophages (Melo, 2009). *Toxoplasma gondii* depends on its host for cholesterol (Portugal et al., 2008). *T. gondii* stores excess esterified cholesterol in intraparasitic LDs (Sonda et al., 2001). Infections involving *T. cruzi* and *T. gondii* show significant correlation between LD formation and production of eicosanoids, specifically PGE_2 (Almeida et al., 2018; Toledo et al., 2016). Other parasites that also induce the accumulation of large LDs in infected macrophages include *Leishmania amazonensis* (Lecoeur et al., 2013) and *Leishmania major* (Rabhi et al., 2012). The prevailing theory is that host lipids from the LDs play an essential role in sustaining successful parasite replication within the parasitophorous vacuole (Toledo et al., 2016).

Various viruses use host LDs as sites of replication (Meester et al., 2011). Hepatitis C virus (HCV) is highly dependent on lipid metabolism in infected cells (Herker and Ott, 2011). The current view is that LDs may serve as assembly platforms for HCV (Herker and Ott, 2012). The viral core protein translocates to the surface of LDs and then recruits viral RNA replication complexes to the ER membranes, which are proximal to LDs, where encapsidation occurs. Mechanistic insight has been gained into the localization, but why HCV requires LDs remains to be elucidated. Zhang and co-workers (2016) showed that when ADRP is overexpressed, it promotes HCV replication, while silencing ADRP promotes HCV assembly. When microRNA 29a is overexpressed in adherent human hepatoma cells (Huh-7) there is an increase in the number of LDs as well as their respective TGs, through inducing the expression of the transcription factor SREBP-1c. Although, there is an increase in lipid droplet numbers it resulted in a reduction of HCV RNA levels in Huh-7 cells (Mamdouh Mahdy et al., 2016). Dengue virus (DENV) also requires LDs for replication as cells treated with fatty acid synthase inhibitors (which block generation of all LDs) attenuates viral replication at the step of assembly and release (Samsa et al., 2009). Recently, Remenyi and co-workers (2017) found

that the NSP3 of Chikungunya virus (CHIKV) associates with LDs, although the significance of this association needs to be further studied. Finally, various viral proteins have been found to interact with LDs, these include µ1 outer capsid protein of reoviruses (Coffey et al., 2006) and agnoprotein of the polyomavirus BK (Unterstab et al., 2010). The biological relevance of these associations remains to be elucidated. Recently, Monson and co-workers (2018) showed that LDs play a role in an efficient early innate host response to viral infection. Huh-7 and HeLa cells with reduced LD content showed a significantly reduced production of type I and III IFN, as well as downstream ISG expression, when infected with Sendai virus. The decrease in the production and expression of type I and III IFNs and ISGs are indicative of an impeded innate immune response.

1.4.7. Association with rotavirus-induced viroplasms

It was shown before that PLINA and ADRP co-localize with rotavirus NSP2 and NSP5 (**Figure 1. 8**) (Cheung et al., 2010). This association between viral proteins and host organelles is a classic example of a virus hijacking a cellular organelle for its own replication. Cheung and co-workers (2010) showed co-sedimentation of viral dsRNAs (genome segment 8 and genome segment 11) with PLINA at low densities of iodixanol gradients, while the proximity of NSP5 with PLINA was demonstrated by fluorescence resonance energy transfer (FRET). The blocking or dispersing of LDs results in significant decreases in both the number and size of viroplasms, while also adversely affecting viral progeny (Cheung et al., 2010; Gaunt et al., 2013a). It has been suggested that the total lipid content increases RV infection and is indicated by an increase of LDs interacting with viroplasms (Gaunt et al., 2013b). Furthermore, work by Gaunt and co-workers (2013a) showed that viral infectivity can be negatively impacted by the inhibition of fatty acid synthesis using a range of approaches, inferring a role for LDs in virus assembly and/or release.
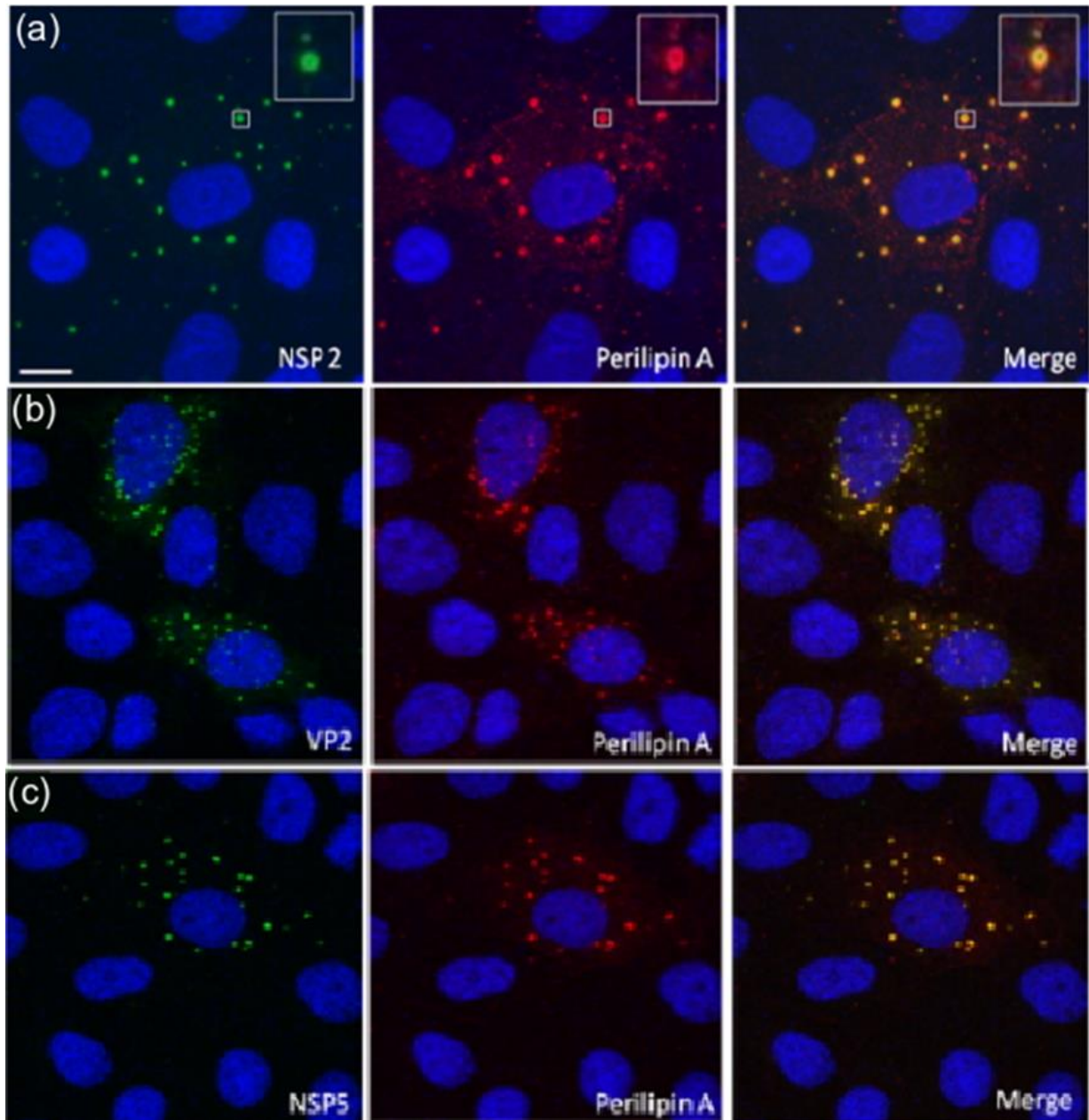
Figure 1. 8. The association of lipid droplets with viroplasm proteins. Confocal microscopy images (CM) of MA104 cells infected with rotavirus obtained 8 h post infection. a) NSP2 (Alexa Fluor 488 (green)) co-localizes with perilipin A (Alexa Fluor 633 (red)). CM images of MA104 cells infected with rotavirus shown at 6 h post infection. b) VP2 and c) NSP5 (Alexa-Fluor 488 (green)). Modified from (Cheung et al., 2010). Permission obtained from publisher.

1.5. Problem statement

It is now well-known that the inhibition of lipid synthesis and LD formation was shown to have deleterious effects on RV replication (Cheung et al., 2010; Gaunt et al., 2013a). Work done by Schie and co-workers (2013) in HepG2 cells showed that when the cells were treated with different amounts of FAs the lipid composition of lipid droplets changed accordingly, while Jin and co-workers (2017) showed the omega3/6 ratio affected FA profiles, antioxidant capacity, haematological characteristics and expression of lipid-related genes. It remains unknown what effect there will be on RV replication when the FA composition of LDs are changed. Furthermore, the role of either saturated or unsaturated FA in RV replication is unknown. This role could be essential in filling gaps with regards to the immune response towards RV infection, as FA and their downstream metabolites are critical immune signallers (Calder and Grimble, 2002; Hwang, 2002), such as eicosanoids (Dennis and Norris, 2015).

1.6. Aim and objectives

The study aims to investigate the effect of fatty acid saturation on rotavirus replication.

Study objectives:

1. To investigate what effect the change in cellular FA composition (neutral lipids and phospholipids), due to the supplementation of FAs at different degrees of saturation, may have on the yield of infectious rotavirus.

2. To investigate the effect of the modulation of FA composition on eicosanoid production and subsequent effects on rotavirus replication.

CHAPTER 2: THE EFFECT OF LIPID SUPPLEMENTATION ON ROTAVIRUS YIELD AND RATE OF REPLICATION

2.1. Introduction

Fatty acids (FAs), which form an integral part of many lipids, are important components of biological compounds and can be used for energy (Tvrzicka et al., 2011). It is well known that cells, which are supplemented with exogenous FAs, take up these FAs and incorporate or metabolise them in the cell (Bolognesi et al., 2013; Popp-Snijders et al., 1986; Stoll and Spector, 1984; Wisnieski et al., 1973). Furthermore, it is also known that FAs can play an immunomodulatory role and that the FA composition in a cell can determine the type and strength of the immune response (Calder, 2008; Damsgaard et al., 2007; de Pablo and Alvarez de Cienfuegos, 2000; Horrobin, 1990; Kelley, 2001). Finally, it has also been shown that various viral infections, including rotavirus (Gaunt et al., 2013b), dengue virus(Heaton et al., 2010), influenza virus(Scheiffele et al., 1999) and hepatitis C virus (Syed et al., 2010) are able to change the lipid composition of cells.

Lipid droplets are major storage sites for lipid esters (Martin and Parton, 2006) and several enzymes for lipid and eicosanoid synthesis associate with LDs (Bozza et al., 2009; Cohen et al., 2004; Martin et al., 2005; Moore et al., 2005; Nakamura et al., 2005; Weller and Dvorak, 1994). In addition, LDs are known to play significant roles in the replication cycle of hepatitis C (Miyanari et al., 2007), dengue virus (Samsa et al., 2009) and rotavirus (RV) (Cheung et al., 2010; Crawford and Desselberger, 2016; Gaunt et al., 2013a). It is unknown if the composition as well as degree of unsaturation of FAs within host cells could have any effect on the replication of RV as in the case of brome mosaic virus (Lee and Ahlquist, 2003) and dengue virus (Zaitseva et al., 2010).

The aim of the research presented in this chapter was to determine what effect saturated and unsaturated FA supplementation will have on the lipid composition of monkey kidney (MA104) cells and the subsequent effect on RV rate of replication.

2.2. Materials and Methods

2.2.1. Cells, culture conditions

African green monkey kidney (MA104) cells (a kind gift from Dr. Ulrich Desselberger, University of Cambridge) were used in all experiments as they are known to be sensitive to RV replication, resulting in high viral yield (Estes et al., 1979). MA104 cells were seeded (~5.2 x 10^6 cells/ml) into five 175 cm^2 culture flasks (Thermo Scientific, USA). The cells were

maintained in growth media: Dulbecco's minimal essential medium (DMEM) (Thermo Scientific, USA) supplemented with 2 % foetal bovine serum (FBS) (Gibco, USA), antibiotic mixture [penicillin (10 000 U/ml), streptomycin (10 000 µg/ml) and amphotericin B (0.25 µg/ml)] (Lonza, Switzerland) and 1 % nonessential amino acids (NEAA) (Lonza, Switzerland). Cells were cultured to 70 – 80 % confluency at 37 °C and 5 % CO_2 in a Steri-Cycle CO_2 incubator (Thermo Scientific, USA).

2.2.2. Supplementation of MA104 cells with fatty acids

MA104 cells were seeded (~5.2 x 10^6 cells/ml) into 175 cm^3 culture flasks and cultured as previously described. MA104 cells were supplemented with FAs when reaching confluency of 60 – 70 % by replacing the medium with growth media containing one of the following, 50 µM stearic acid (18:0; SA) (Sigma-Aldrich, USA), γ-linolenic acid (18:3; GLA, omega 6) (Sigma-Aldrich, USA) or oleic acid (18:1; OA, omega 9) (Sigma-Aldrich, USA) for 24 h according to Tanaka and co-workers (2007) (**Figure 2. 1**). The FAs were added to the medium as an albumin (Albumin fraction VI (Roche, Germany) complex with a molar ratio of 3:1 (Tanaka et al., 2001a). Briefly, FAs, stored in ethanol, were dried under N_2 gas and dissolved in albumin by sonication, where after the complex was filter-sterilized before addition to growth media. An equivalent amount of albumin was added to 60 – 70 % confluent cells as albumin control.
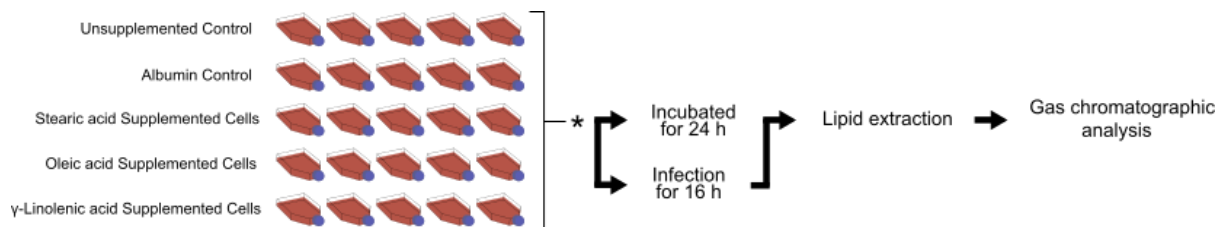
Figure 2. 1. Schematic representation of supplementation. MA104 cells were grown to 60 – 70 % confluence, after which the medium was replaced with growth medium containing 50 µM of either stearic acid (SA), oleic acid (OA) or γ-linolenic acid (GLA). After 24 h of supplementation, fatty acids was extracted and submitted for gas chromatographic analysis. One biological replicate consisted of five 175 cm^2 flasks.

2.2.3. Infection of MA104 cells with rotavirus SA11

All cells were infected with the prototype rotavirus (RV) strain, simian agent (SA11) (Mlera et al., 2013). Rotavirus SA11 was activated at 37 °C for 30 min using 10 µg/ml trypsin IX (Sigma-Aldrich, USA). Supplemented MA104 cells were infected with the SA11 strain at a multiplicity of infection (MOI) of 1 and incubated in infection media: serum-free DMEM containing antibiotic mixture, NEAA and supplemented with 1 µg/ml trypsin IX s for 45 min on an orbital rocker (Bench Rocker 2D). After infection, the cells were washed once with 0.01 M, pH 7.4

phosphate buffered saline (Sigma-Aldrich, USA) (PBS) and then incubated for 15 h and 15 min in infection media. Controls consisted of uninfected cells as well as unsupplemented infected cells and were processed in parallel under the same conditions. <mark>Working practises can be found in appendix B.</mark>

2.2.4. Viral titrations using 50 % tissue culture infective dose (TCID$_{50}$)

MA104 cells were seeded (0.05 x 10^6 cells/ml) into 24-well culture plates (Thermo Scientific, USA) and cultured to 70 – 80 % confluency and infected as previously described. Infected MA104 cells were freeze-thawed three times, after which the cell debris was removed through centrifugation (Yingtai TD5) at 4000 x g for 10 min to obtain viral lysate. The MA104 cells were washed three times with PBS and serial dilutions (10^{-4} to 10^{-7}) from the viral lysate were added to the MA104 monolayers and incubated for 45 min. After infection, the cells were washed with PBS and infection media was added to the wells. The infected MA104 monolayers were incubated at 37 °C and 5 % CO$_2$ for 5 days and titres were read on day five. TCID$_{50}$ titres were calculated for the duplicate preparations as previously described (Reed & Muench, 1938).

2.2.5. Lipid analysis

Supplemented and infected cells were washed three times with PBS. The cells were scraped off after 24 h incubation with FA-supplemented media or after supplementation and infection for an additional 16 h and centrifuged at 1500 x g for 4 min. The harvested cells were frozen overnight at -20 °C, followed by freeze-drying overnight. Total lipids were extracted using chloroform/methanol (2:1) (Folch et al., 1957). Various controls (i.e. supplemented uninfected cells, unsupplemented infected cells and unsupplemented uninfected cells) were processed in parallel under the same conditions. Lipids were fractionated by the use of 500 mg amino propyl silica cartridges (Varian, USA) according to the method of Bossier and Scow (Bossio and Scow, 1998). Briefly, cartridges were conditioned with 2 ml chloroform, followed by the addition of the extracted lipid samples to the cartridges. The neutral lipids were eluted with 5 ml chloroform, while the glycolipids and phospholipids were eluted with 10 ml acetone and 5 ml methanol, respectively. The eluates were collected and dried under N$_2$ at 37 ℃. The lipids were dissolved in chloroform (Sigma-Aldrich, USA) and transestrified with trimethylsulfonium hydroxide (Sigma-Aldrich, USA). Fatty acid methyl esters (FAMEs) were analysed using a Varian 430 flame ionization gas chromatograph (GC), with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thicknesses). Analysis was performed using an initial isothermic period (40 °C for 2 minutes). Thereafter, temperature was increased at a rate of 4 °C/minute to 230°C. Finally, an isothermic period of 230 °C for 10 minutes followed. FAMEs in n-hexane (1µl) were injected into the column using a Varian CP

8400 autosampler. The injection port and detector were both maintained at 250 °C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Galaxy Chromatography Software recorded the chromatograms. Fatty acid methyl esters were identified by comparing the retention times of FAME peaks from samples with those of standards obtained from Supelco (Supelco 37 Component Fame Mix 47885-U, Sigma-Aldrich, USA). The relative percentage of individual FAs was determined by dividing the peak area of each FA by the total peak area.

2.2.6. Determination of SA11 replication rate in supplemented MA104 cells

MA104 cells were seeded (~0.7 x 10^6 cells/ml) in 25 cm^2 cell culture flasks and supplemented and subsequently infected with SA11 as previously described for 0, 6, 16, 24, 30 and 48 h in triplicate. Infected MA104 cells were freeze-thawed three times, after which the media was clarified through centrifugation (Yingtai TD5) at 4000 x g for 10 min to obtain viral lysate. MA104 cells were seeded in 96 well plates (Thermo Scientific, USA) and allowed to replicate in DMEM containing 2 % FBS, antibiotic mixture and NEAA. $TCID_{50}$ was done as explained in section 2.2.4 using viral dilutions from 10^{-5} to 10^{-12}. To ensure that RV yield was not due to variation in cell number before infection, supplemented cells were counted to ensure normalisation. Briefly, supplemented cells were trypsinized (Lonza, Switzerland) for 5 min to detached cells from the 25 cm^2 cell culture flask. Cells were pelleted by centrifugation 800 x g for 5 min. The cells were resuspended in 1 ml DMEM medium and mixed in a 1:1 ratio with Trypan Blue (Thermo Scientific, USA). Viable cells were counted and the number of cells were determined using a cell-counting chamber (Neubauer) and inverted light microscope (Nikon Eclipse TS100). Briefly, the number of unstained cells (living cells) were counted in four sets of 16 squares. The average of the four sets was calculated and multiplied with 10^4 and the dilution factor and expressed as cells/ml.

2.2.7. Determination of yield of SA11 infectivity in supplemented MA104 cells

MA104 cells were seeded (~0.7 x 10^6 cells/ml) into 25 cm^2 cell culture flasks, supplemented and subsequently infected with SA11 as previously described for 16 h. Infected MA104 cells were freeze-thawed three times, after which the media was clarified through centrifugation (Yingtai TD5) at 4000 x g for 10 min to obtain viral lysate. MA104 cells (0.05 x 10^6 cells/ml) were seeded in 24-well culture plates and incubated in DMEM containing 2 % FBS, antibiotic mixture and NEAA. $TCID_{50}$ was carried out as explained in section 2.2.4 using viral dilutions from 10^{-5} to 10^{-12}. Data was normalised as previously described in 2.2.6.

2.2.8. Statistical analysis

All experiments were performed at least in triplicate unless stated otherwise. In cases where three or more values were obtained, the ANOVA test (Fisher, 1925) and the Tukey-Kramer (Tukey, 1949) ad hoc test were performed to determine the significance of the data sets unless otherwise stated. A p value of 0.05 or less was considered significant.

2.3. Results

2.3.1. Fatty acid supplementation modulates the fatty acid composition of MA104 cells

To determine the change in FA composition of MA104 cells after supplementation, GC-analysis was carried out. **Figure 2. 2** shows the relative FA percentage of MA104 cells after supplementation with albumin, SA, OA, GLA. The neutral lipid (**Figure 2. 2A**) and phospholipid fraction (**Figure 2. 2B**) of MA104 cells supplemented with albumin, showed no significant difference in any of the FAs of MA104 cells. Although supplementation of MA104 cells with SA had no significant effect on the relative percentage of SA in either the neutral lipids or phospholipids, it did lead to a significant increase in nervonic acid and linolelaic acid, with a concomitant decrease in palmitic acid, OA and α-linolenic acid in the neutral lipids, as well as palmitic acid in the phospholipid fraction. As expected, supplementation of MA104 cells with OA significantly increased the relative percentage of OA in both the neutral lipids and phospholipids. Oleic acid supplementation also significantly increased the relative percentage of α-linolenic and dihomo-γ-linolenic acid, but decreased palmitic, stearic, arachidic and nervonic acid in the neutral lipid fraction (**Figure 2. 2A**). In addition, OA supplementation also significantly decreased palmitic acid in the phospholipid fraction (**Figure 2. 2B**). Supplementation of MA104 cells with GLA significantly increased the relative percentage of GLA and erucic acid in the neutral fraction (**Figure 2. 2A**). Concerning the phospholipid fraction, GLA supplementation significantly increased the relative percentage of both GLA and arachidonic acid, while significantly decreasing OA (**Figure 2. 2B**). Thus, from this data it can be clearly seen that supplementation of MA104 cells with different FAs, leads to compositional changes within the lipid profile of MA104 cells.
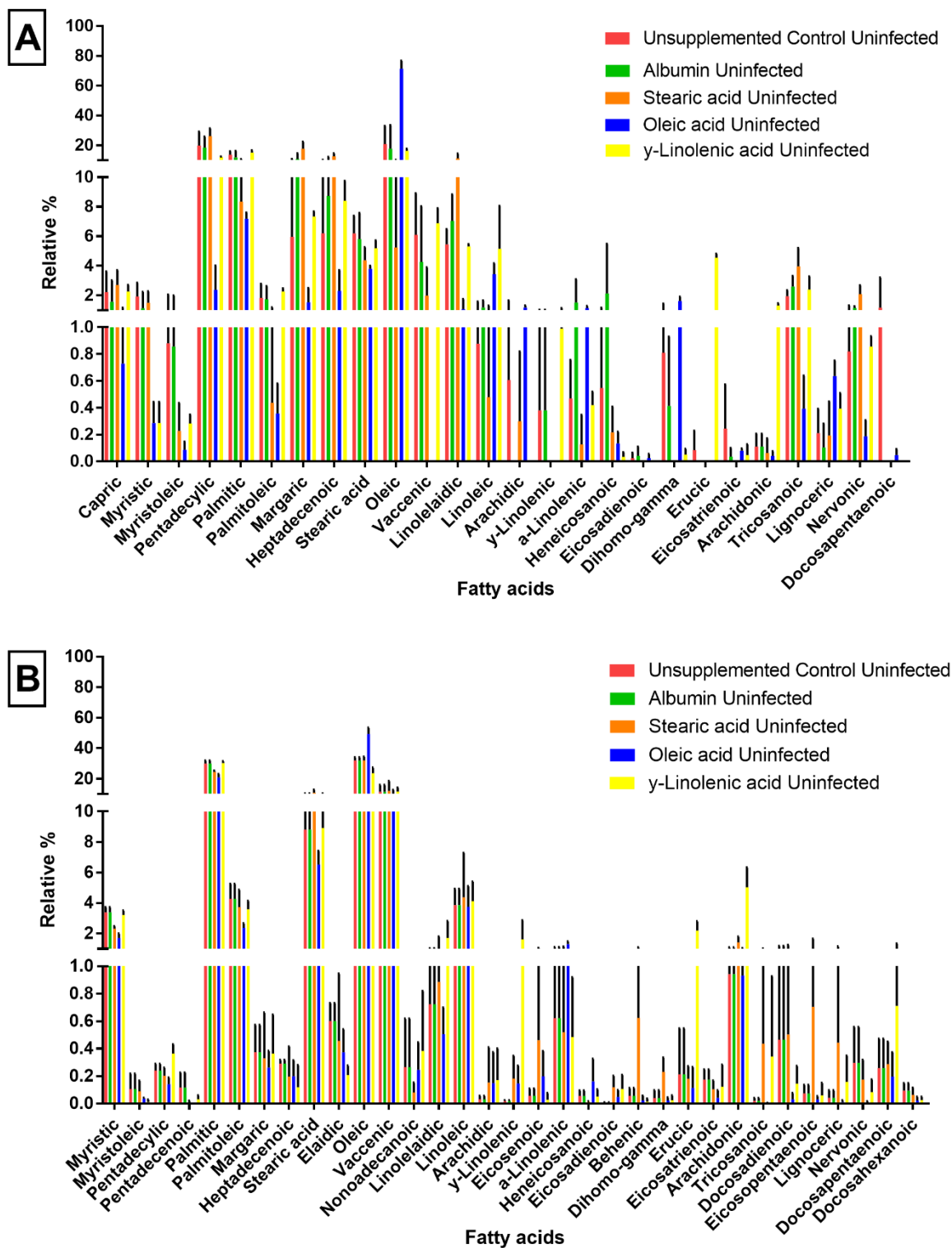
Figure 2. 2. The relative percentage of fatty acids following supplementation of MA104 cells with various fatty acids. Fatty acid profiles of MA104 cells after incubation for 24 h at 37 °C in the absence and presence of one of the following: 50 µM stearic acid, oleic acid, γ-linolenic acid. (A) The percentage fatty acids in the neutral lipids. (B) The percentage fatty

acids in the phospholipids. <mark>Values represent the mean of three biological replicates and the standard deviation is indicated</mark> as black bars.

The omega-6/omega-3 ratio of supplemented MA104 cells was calculated, to determine if the inflammatory response in supplemented cells would be pro- or anti-inflammatory. **Figure 2. 3** indicates that supplementation of MA104 cells with GLA significantly increased the omega-6/omega-3 ratio in both neutral and phospholipid fraction.
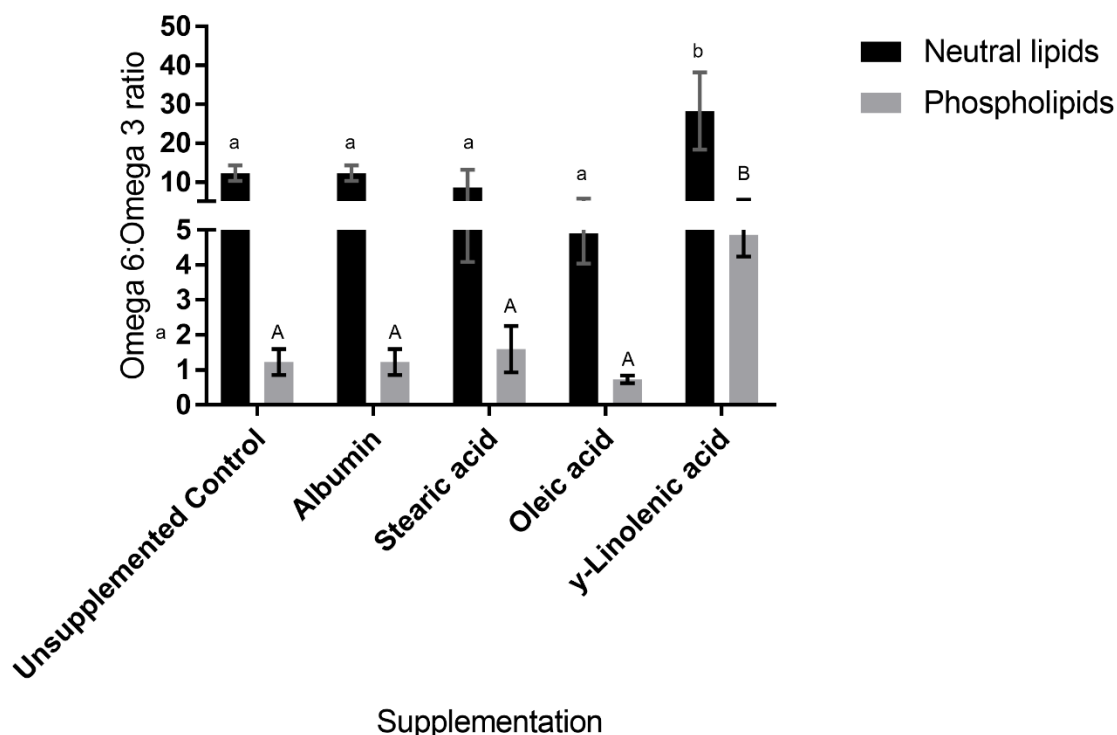
Figure 2. 3. The omega-6/omega-3 fatty acid ratio of supplemented MA104 cells. The supplementation of MA104 cells with GLA was the only supplementation that had any significant effect on the omega-6/omega-3 ratio. <mark>Values represent the mean of three biological replicates. Standard deviation is indicated by error bars.</mark> Uppercase letters indicate significant difference from lowercase letters.

2.3.2. Rotavirus modulates the fatty acid composition of MA104 supplemented cells

GC-analysis was performed to determine the change in FA composition of MA104 cells after supplementation and subsequent infection with RV. **Figure 2. 4** shows the relative FA percentage of MA104 cells after supplementation with albumin, SA, OA, GLA and subsequent infection with RV. The neutral lipid (**Figure 2. 4A**) and phospholipid fractions (**Figure 2. 4B**) of MA104 cells supplemented with albumin and subsequent infection with RV, showed no significant difference compared to infected unsupplemented MA104 cells. Although

supplementation of MA104 cells with SA and subsequent infection with RV had no significant effect on the relative percentage of SA in either the neutral lipids or phospholipids, it did lead to a significant decrease in myristic and vaccenic acid in the neutral lipids, with no effect in the phospholipid fraction compared to infected unsupplemented MA104 cells. As expected, supplementation of MA104 cells with OA and subsequent infection with RV significantly increased OA within the phospholipids. Oleic acid supplementation and subsequent RV infection also significantly increased the relative percentage of myristoleic, α-linolenic and heneicosanoic acid, but decreased margaric, linolelaidic, and nervonic acid in the neutral lipid fraction (**Figure 2. 4A**). In addition, OA supplementation and subsequent RV infection significantly decreased palmitoleic acid in the phospholipid fraction (**Figure 2. 4B**). Supplementation of MA104 cells with GLA and subsequent infection with RV had no effect on the relative percentage of GLA or AA acid in the neutral fraction (**Figure 2. 4A**). Concerning the neutral lipid fraction, GLA supplementation and subsequent RV infection significantly increased the relative percentage of heneicosanoic acid and eruric acid, while significantly decreasing myristoleic, pentadecylic, vaccenic and nervonic acid. In the phospholipid fraction GLA supplementation and subsequent RV infection significantly decrease the relative percentage of OA, while significantly increasing the amount of AA, eruric and docosahexanoic acid (**Figure 2. 4B**). Thus, from this data it can be clearly seen that infection of supplemented MA104 cells with RV, leads to unique compositional changes within the lipid profile of infected and supplemented MA104 cells.
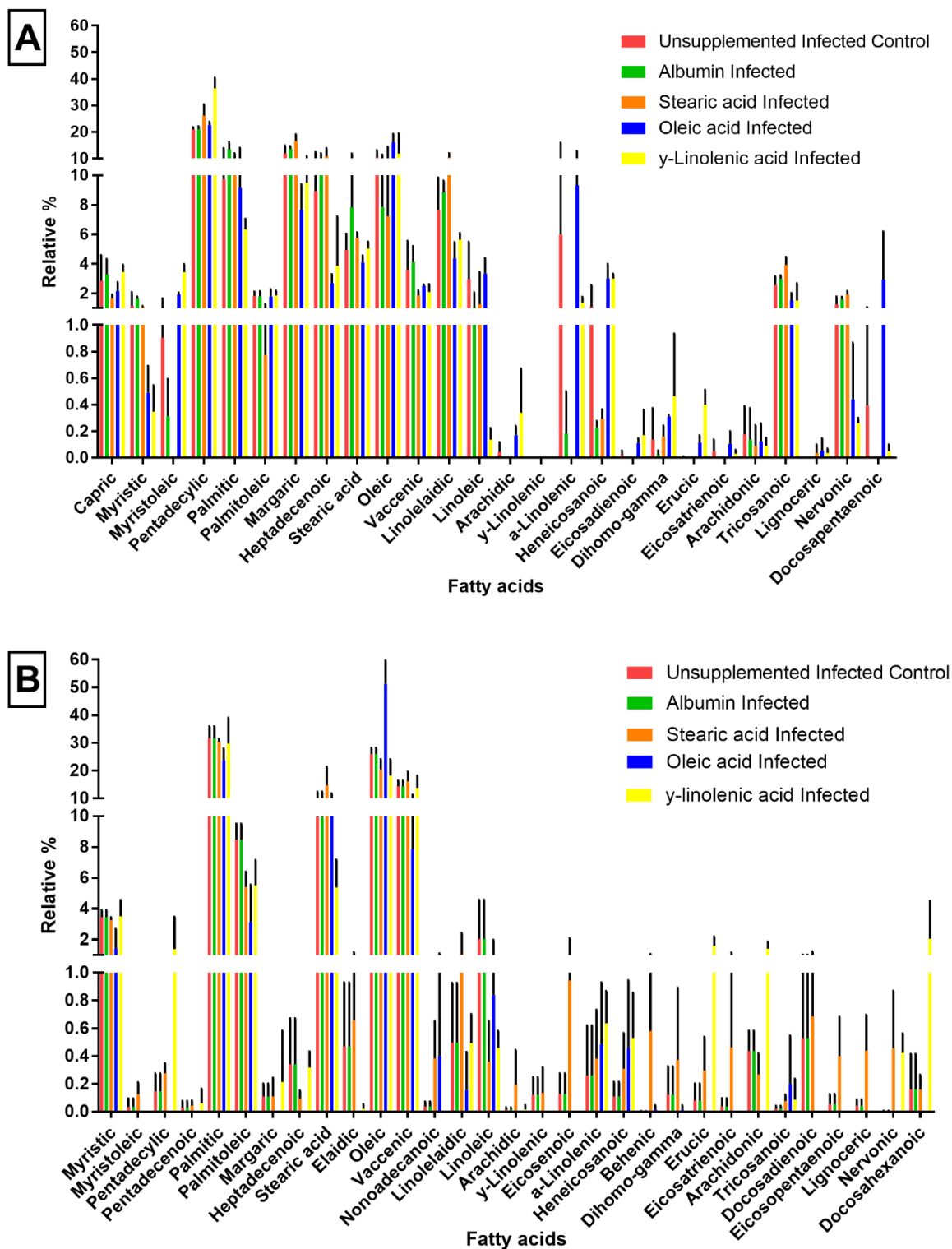
Figure 2. 4. The relative percentage of fatty acids in rotavirus infected and supplemented MA104 cells. Fatty acid profiles of MA104 cells after incubation for 24 h at 37 °C and subsequent infection with SA11 (MOI = 1) for 16 h in the absence and presence of one of the following: 50 μM stearic acid, oleic acid, γ-linolenic acid. (A) The percentage fatty acids

in the neutral lipids. (B) The percentage fatty acids in the phospholipids. <mark>Values represent the mean of three biological replicates and the standard deviation is indicated</mark> <mark>as black bars</mark>.

The omega-6/omega-3 ratio of supplemented MA104 cells was calculated, to determine if the inflammatory response in supplemented cells would be pro- or anti-inflammatory. **Figure 2. 3** indicates that supplementation of MA104 cells with SA and the subsequent infection with RV significantly decreases the omega-6/omega-3 ratio within the phospholipid fraction only, while both OA and GLA supplementation and subsequent infection with RV significantly decreased the omega-6/omega-3 ratio in both neutral and phospholipid fraction. `
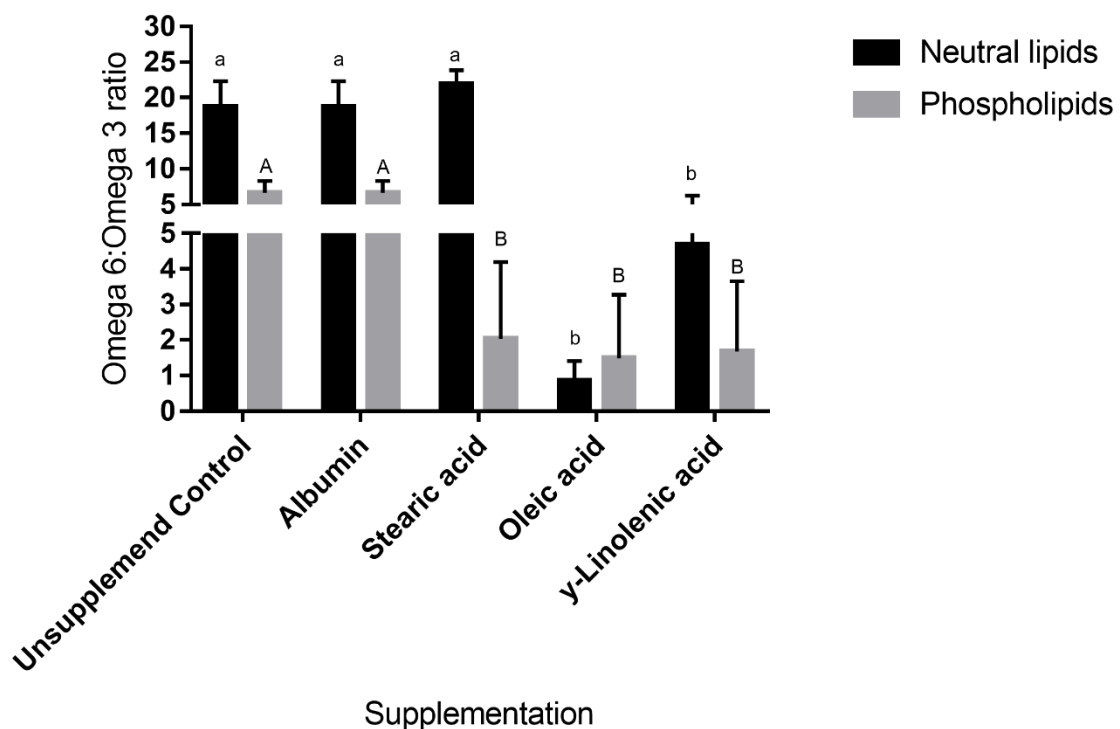
Figure 2. 5. The omega-6/omega-3 fatty acid ratio of supplemented infected MA104 cells. The supplementation of MA104 cells with SA and subsequent infection with RV significantly decrease the omega-6/omega-3 ratio within the phospholipid fraction, while both OA and GLA supplementation and subsequent RV infection significantly decreased omega-6/omega-3 ratio in the phospholipid and neutral lipid fraction. <mark>Values represent the mean of three biological replicates. Standard deviation is indicated by error bars</mark>. Uppercase letters indicate significant difference from lowercase letters.

2.3.3. Comparison between selected fatty acids in MA104 supplemented uninfected cells and MA104 supplemented and RV infected cells.

To determine whether rotavirus has an add-on modulatory effect on the lipid profile of supplemented MA104 cells, the lipid profiles of supplemented MA104 cells were compared to supplemented and infected MA104 cells. **Figure 2. 6** shows only the FAs in supplemented MA104 cells that were significantly influenced by subsequent RV infection. Of particular importance is the fact that, regardless of supplementation, rotavirus was able to significantly decrease the relative percentage of OA within the neutral lipid fractions (**Figure 2. 6A$_1$, B$_1$ and C$_1$**), while significantly decreasing AA within the phospholipid fraction (**Figure 2. 6 A$_2$, B$_2$ and C$_2$**). Furthermore, supplementation of MA104 cells with OA (**Figure 2. 6 B$_1$ and B$_2$**) or GLA (**Figure 2. 6 C$_1$ and C$_2$**) followed by RV infection, leads to more fatty acids being affected when compared to the unsupplemented and infected control (**Figure 2. 6 A$_1$ and A$_2$**). In addition, the neutral lipid fraction of both OA (**Figure 2. 6 B$_1$**) and GLA (**Figure 2. 6 C$_1$**) supplemented and infected MA104 cells showed more fatty acids being affected, compared to the phospholipid fraction of OA (**Figure 2. 6 B$_2$**) and GLA (**Figure 2. 6 C$_2$**) supplemented and infected MA104 cells. Oleic acid supplementation and subsequent RV infection increased the relative percentage of α-linolenic acid, while decreasing relative percentage of dihomo-γ-linolenic acid within the neutral lipids. The same increase in the relative percent of α-linolenic acid is seen when GLA supplemented MA104 cells are infected with RV, while γ-linolenic acid is decreased.
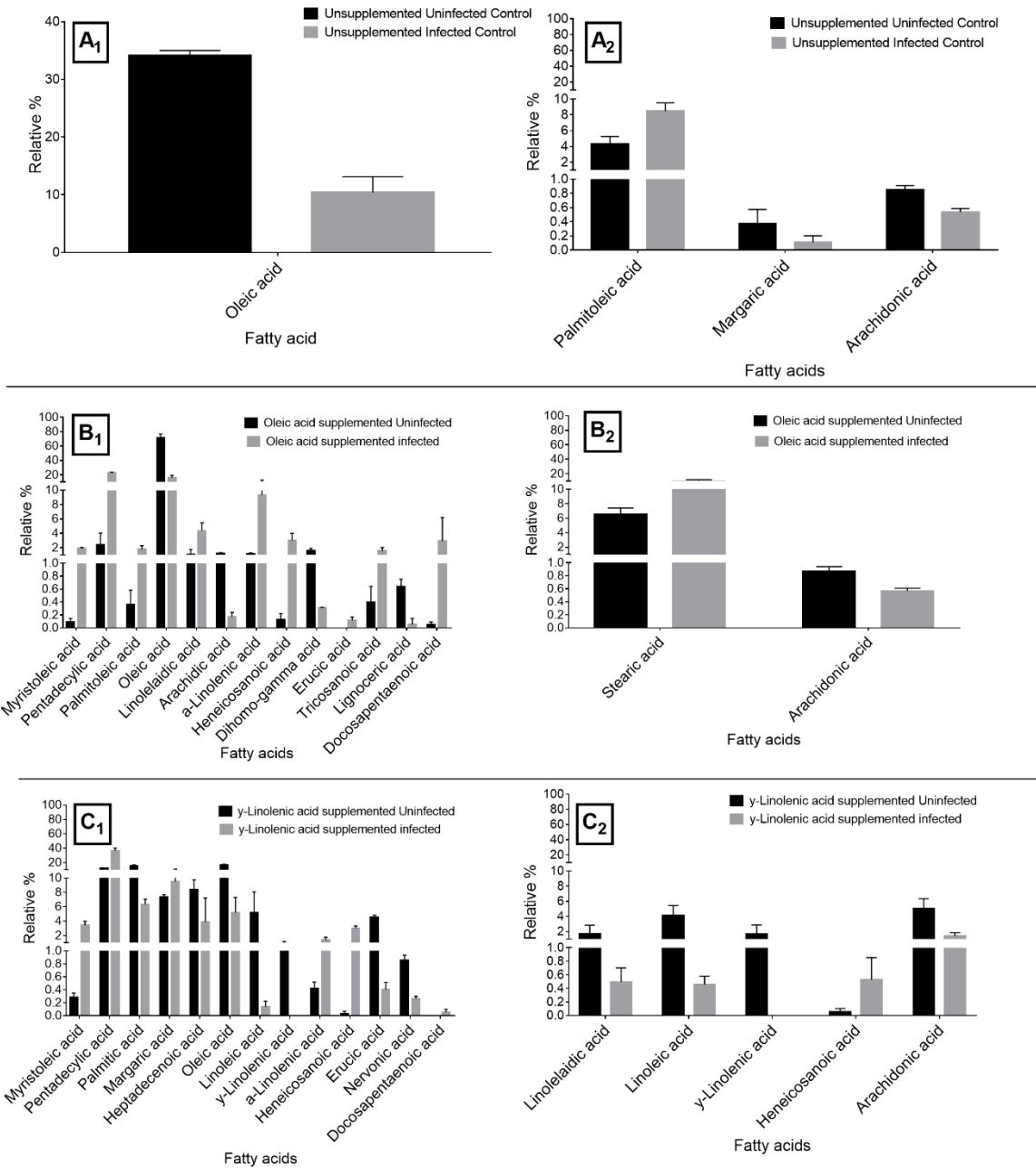
Figure 2. 6. Comparison between the effects of only FA supplementation or FA supplementation and subsequent infection with SA11 on the lipid profile of MA104. Panel A shows the lipid profile of unsupplemented and infected cells, while B is OA supplemented and infected cells and C is GLA supplemented and infected cells. 1 indicates the neutral lipid fraction while 2 indicated the phospholipid fraction. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars.

2.3.4. Rate of rotavirus SA11 replication in supplemented MA104 cells

To determined the rate of replication for SA11, viral titrations in the form of tissue culture infectious doses (TCID$_{50}$) were determined at 0, 6, 16, 24, 30 and 48 h post infection. It should be noted that the number of cells did not differ significantly from the control and thus no normalization of the data was performed (**Figure 2. 7**). Growth curves of SA11 in supplemented MA104 cells at a MOI of 1 is shown in **Figure 2. 7**. Irrespective of supplementation, the maximum titres of SA11 produced in supplemented MA104 cells were higher than the input titre. Supplementation of MA014 cells with GLA yielded the greatest fold-increase (3-fold) in maximum titres when compared to the input titre, followed by OA (2-fold) when compared to the fold-increase in unsuppplemented cells. Supplementation of MA104 with albumin and SA had no significant effect in the fold-increase when compared to the control. Supplementation of MA014 cells with GLA ($1.08 \times 10^{10} [\pm 2.3 \times 10^{9}]$ pfu/ml) showed the greatest significant increase in viral yield at 16 h post infection when compared to the control ($2.33 \times 10^{7} [\pm 1.9 \times 10^{7}]$ pfu/ml). This significant increase in yield was also observed in cells supplemented with OA ($7.59 \times 10^{8} [\pm 2.8 \times 10^{8}]$ pfu/ml), while albumin- ($2.78 \times 10^{7} [\pm 2.7 \times 10^{6}]$ pfu/ml) and SA11-supplemented ($3.25 \times 10^{7} [\pm 1.7 \times 10^{7}]$ pfu/ml) cells showed no significant increases. The supplementation of MA104 cells with GLA appears to produced the greatest yield of SA11 16 h post infection, while all other supplementations reached greatest yield only after 30 h post infection. The replication curves thus clearly implicate unsatuatured fatty acids, and in particular GLA, in the replication of SA11.
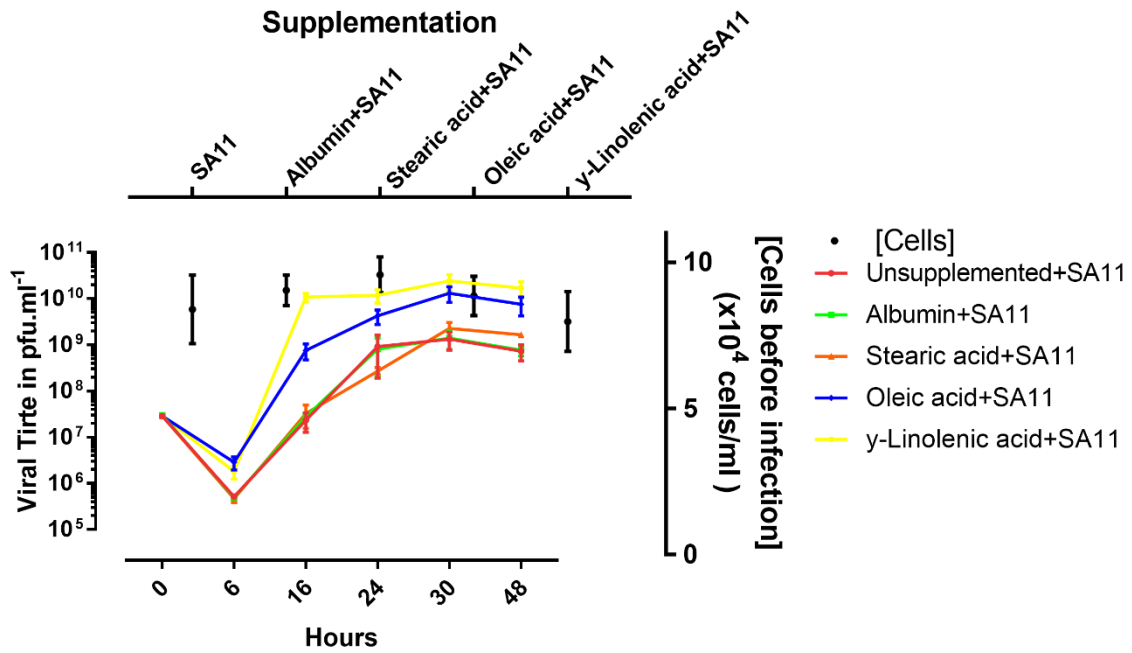
Figure 2. 7. The rate of rotavirus SA11 replication in supplemented and unsupplemented MA104 cells. MA104 cells were supplemented with 50 µM of each respective FA for 24h, where after supplemented MA104 cells were infected for 16 h. $TCID_{50}$ were used to determine the rate of viral replication at several time points post infection. Bottom x-axis pertains to rotavirus rate of replication, while the top x-axis pertains to cells concentration before infection. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars.

2.3.5. Viral yield of Rotavirus SA11 in supplemented MA104 cells

To determine the viral yield of SA11, viral titrations were determined at 16 h post infection. It should be noted that the number of cells did not differ significantly from the control and thus no normalisation of the data was required (**Figure 2. 8**). The supplementation of MA014 cells with unsaturated FAs led to significant increases in the yield of RV 16 h post infection (**Figure 2. 8**). Supplementation of MA104 cells with albumin or SA had no effect on the viral yield when compared to the control. Supplementation of MA104 cells with GLA led to an approximate 3-fold increase in viral yield when compared to the unsupplemented control. In addition to GLA, supplementation of MA104 cells with OA led to an approximate 2-fold increase in viral yield. Thus, supplementation with unsaturated FAs increased the yield of SA11 16 h post infection, while supplementation with albumin and SA appear to have no effect on viral yield.
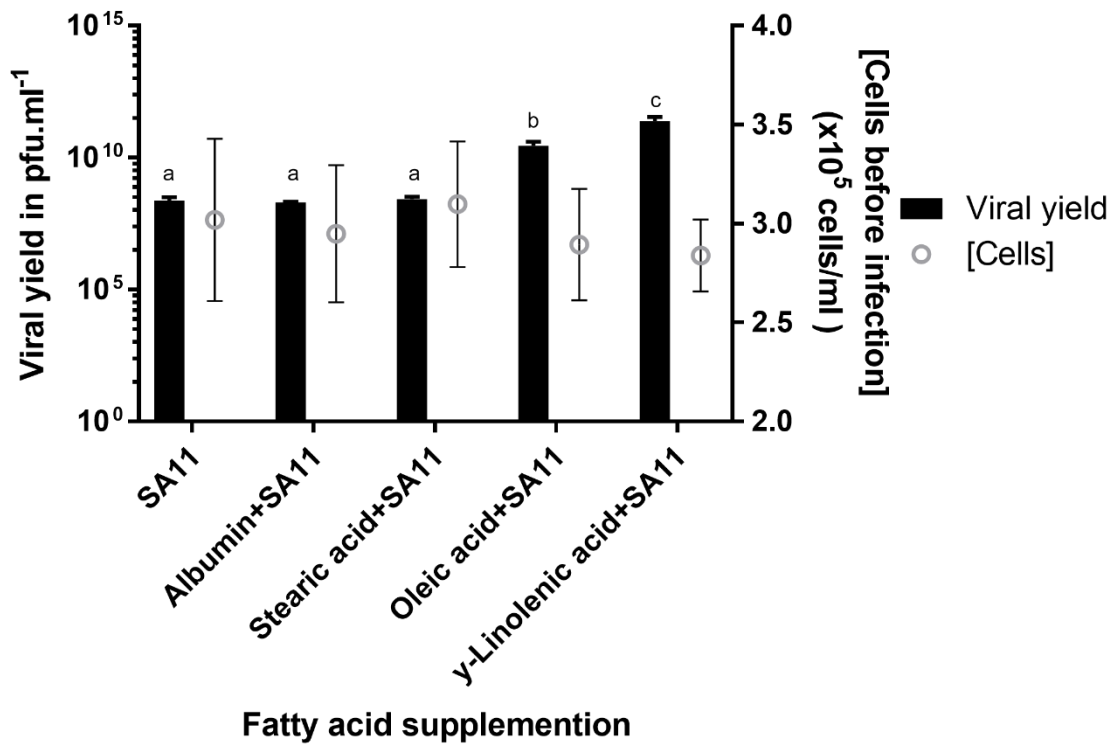
Figure 2. 8. The viral yield of SA11 at 16 h in supplemented and control MA104 cells.
MA104 cells were supplemented with 50 μM of each respective FA for 24h, where after supplemented MA104 cells were infected for 16 h. $TCID_{50}$ were used to determine the yield of virus 16 h post infection. Primary y-axis pertains to rotavirus yield, while the secondary y-axis pertains to cells concentration before infection. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars.

2.4.   Discussion

Fatty acids play diverse roles in immunity. They influence the physical and functional properties of cellular membranes, serve as signalling molecules and regulators of gene expression and are important sources of energy (Calder, 2003, 2006; Miles and Calder, 1998; Yaqoob, 2003). Here we have shown by GC analysis and $TCID_{50}$ that supplementation of MA104 cells with FAs and subsequent infection with SA11 change the lipid composition of MA104 cells. Gas chromatography data showed that supplementation of MA104 cells with FAs of varying degrees of unsaturation can change lipid composition as seen with other cells (Dias et al., 1992; Li et al., 2006; Popp-Snijders et al., 1986; Spector and Yorek, 1985). Supplementation of MA104 cells with SA caused no significant increase in the relative amount of SA, possibly due to the metabolism of SA (Sampath and Ntambi, 2005) (**Figure 2. 9**). The decrease in palmitic acid may be explained by the fact that it is the precursor for SA, and an abundance of SA would prevent the de novo synthesis of palmitic acid. In addition, SA can be converted to OA, which in turn can be converted to nervonic acid, possibly explaining the decrease in OA and the increase in nervonic acid observed.
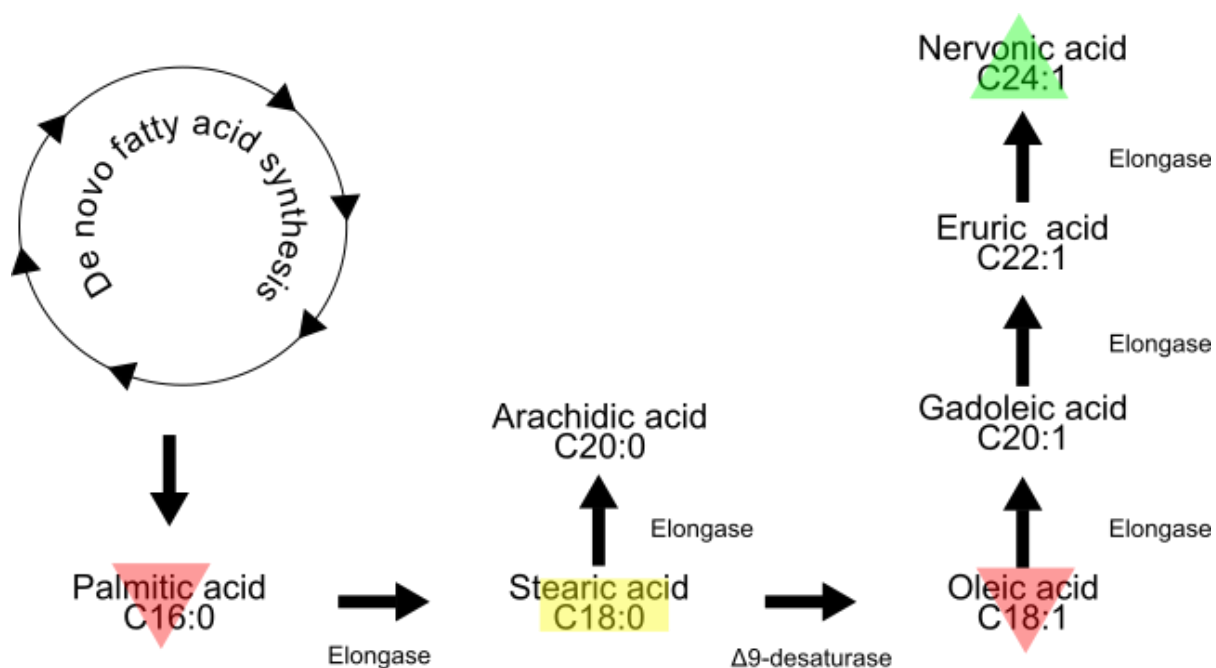
Figure 2. 9. The effect of stearic acid supplementation on the lipid fraction of MA104 cells. Stearic acid had no effect on the relative amount of stearic acid within supplemented cells within either the neutral or the phospholipid fraction, while decreasing the relative amount of palmitic (both lipid fractions) and oleic acid (neutral lipid fraction), and increasing the relative amount of nervonic acid (neutral lipid fraction).

The ease with which OA is taken up from the media by cells, can account for the high relative percentage of OA found within MA104 cells after OA supplementation (Bruce and Salter, 1996) **(Figure 2. 10)**. The decrease in palmitic and SA may also be explained by the fact that OA is a downstream product of these fatty acids and an excess of OA could inhibit the *de novo* production of palmitic acid and thus decrease the amount of SA produced (Priore et al., 2017). The decrease in arachidic acid is explained by the decrease in SA, while the cause of the decrease in nervonic remains unknown. Lastly, supplementation of GLA increased the percentage of GLA and AA and was expected due to the metabolism of GLA (Sergeant et al., 2016) (**Figure 2. 11**).
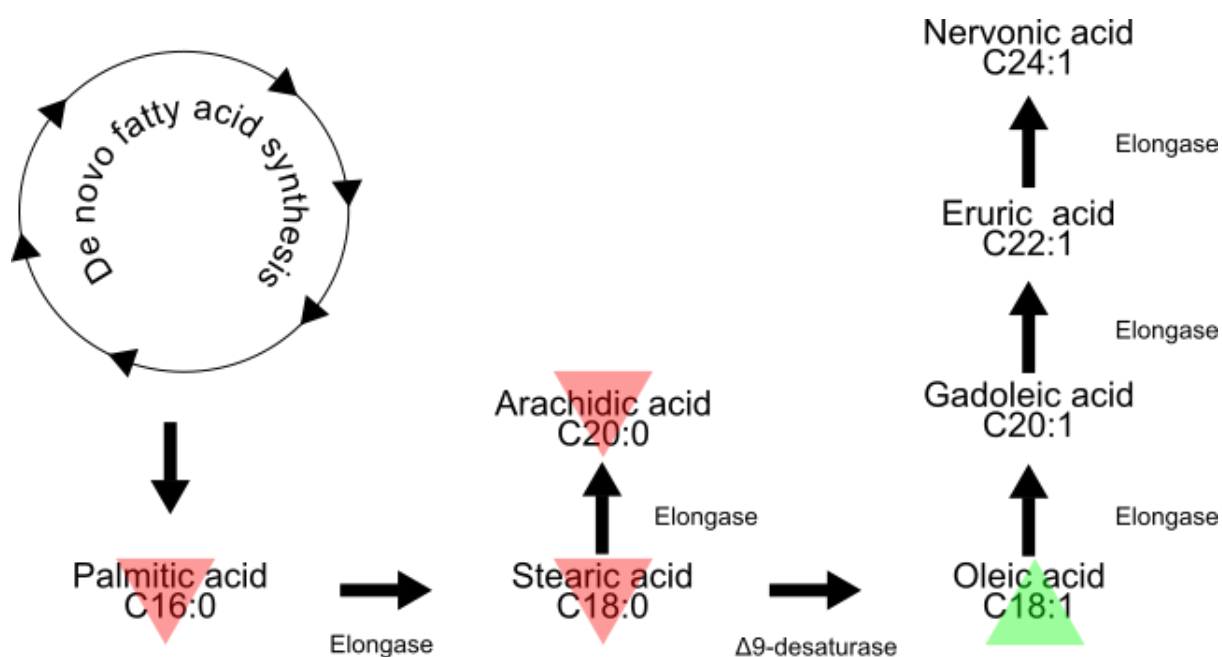
Figure 2. 10. The effect of oleic acid supplementation on the lipid fraction of MA104 cells. Oleic acid increased in both lipid fractions during OA supplementation. The relative amount of palmitic, stearic and arachidic acid were significantly decreased in the neutral lipid fraction.
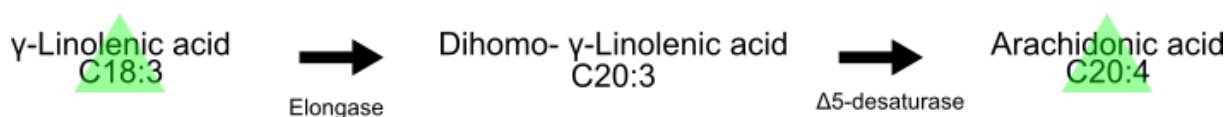
Figure 2. 11. The effect of γ-linolenic acid supplementation on the lipid fraction of MA104 cells. Supplementation of MA104 cells with γ-linolenic acid increased the relative amount of γ-linolenic acid (both lipid fractions) fractions and arachidonic acid (phospholipid fractions) in supplemented MA104 cells.

Infection of supplemented MA104 cells with SA11 induced further changes on the lipid composition of MA104 cells. Data indicates that SA11 can decrease the relative percentage of OA within the neutral lipids, while also decreasing the relative percentage of AA within the

phospholipids irrespective of supplementation. Furthermore, SA11 infection appears to modulate lipids that are precursors to immune signalling molecules in supplemented MA104 cells. It increased the relative percentage of α-linolenic acid, while decreasing the relative percentage of γ-linolenic and dihomo-γ-linolenic acid. This data is in partial concurrence with work done by Gaunt and workers (2013b) who saw that RV was able to induce changes within the lipid profile of MA104 cells. In contrast to their work, we looked at specific fatty acids within the lipid profile, while they only looked at changes within lipid classes. The decrease in GLA and AA is an interesting observation and may be due to the production of PGE_2 (Park et al., 2006) (a immunomodulatory signalling enzyme (Kalinski, 2012)), during RV infection (Yamashiro et al., 1989; Zijlstra et al., 1999). It has been shown that viruses, such as respiratory syncytial virus, are capable of inducing selective changes in the lipid composition of membrane rafts during infection (Yeo et al., 2009). Other viruses, such as cytomegalovirus (Munger et al., 2008) and herpes simplex virus (Blackham et al., 2010), are known to change the biosynthesis, degradation and transport of intracellular lipids during viral infection.

The omega-6/omega-3 ratio was significantly changed within both the neutral lipid fraction and phospholipid fraction when MA104 cells were supplemented with GLA (omega 6). Interestingly, infection of supplemented MA104 cells with SA11 decreased the omega-6/omega-3 ratio in the phospholipid fractions of SA, OA and GLA supplemented cells, while the 6/omega-3 ratio was decreased within the neutral lipid fraction of OA and GLA supplemented cells. Omega-6 FAs result in pro-inflammatory effects, while omega-3 FAs result in anti-inflammatory effects (Calder, 2005). When the omega-6/omega-3 ratio is decreased (e.g. by supplementation of omega-3 fatty acids), the effect on the host depends on the specific infection (Husson et al., 2016). Several studies have shown that omega-3 supplementation of the host during bacterial infections (*Streptococcus pneumonia*, *Pseudomonas aeruginosa, Escherichia coli*), led to a reduction in inflammation which limits the amount of tissue damage due to infection (Myles et al., 2014; Saini et al., 2010, 2011; Tiesset et al., 2011). During influenza virus (Byleveld et al., 1999; Schwerbrock et al., 2009) and herpes simplex virus infection (Courrèges and Benencia, 2002), omega-3 FA supplementation is detrimental towards the host, as it affects immune cell responses and antiviral cytokines. In contrast, enrichment of the diet with omega-3 FAs had no effect during vaccinia virus infection (Jones and Roper, 2017). It was also noted that the beneficial/detrimental effect of omega-3 FAs was dose-dependent, and too high a concentration could switch from beneficial effects (limiting tissue damage) to detrimental (being immunosuppressive) effects (Husson et al., 2016). We were unable to find any studies during which the host was supplemented with omega-6 FAs and subsequently subjected to pathogenic infection.

Using viral titrations, it was shown that the supplementation of MA104 cells with FAs affect the replication of rotavirus SA11. Supplementation with unsaturated FAs, OA and specifically GLA, increased the rate of replication and yield of the virus. By using fatty acid synthase inhibitors, such as 5-tetradecyloxy-2-furoic acid (TOFA) and 4-methylene-2-octyl-5-oxotetra-hydrofuran-3-carboxylic acid (C75), the replication of several viruses such as rotavirus (Gaunt et al., 2013a) vaccinia (Greseth and Traktman, 2014), dengue (Gullberg et al., 2015) and respiratory syncytial virus (Ohol et al., 2015) have been negatively impacted. The inhibition is believed to either change the membrane fluidity in such a way that it impedes viral replication or that it affects the production of energy via β-oxidation for viral assembly. During HIV infection, long chain polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, have been shown to reduce the spread of HIV between cells (Hsu, 2009).

The observation that RV decreases the relative percentage of AA in GLA supplemented cells, while GLA supplementation significantly increases the rate of replication and yield of RV is interesting. It is well known that GLA can be converted to AA and then subsequently to PGE_2 (Park et al., 2006), which can modulate the type and strength of the immune response (Kalinski, 2012). Thus determining the levels of PGE_2 during RV infection following supplementation with saturated and unsaturated fatty acids will be investigated in the following chapter.

CHAPTER 3: EICOSANOID PRODUCTION AFTER LIPID SUPPLEMENTATION AND ROTAVIRUS INFECTION

3.1. Introduction

Eicosanoids are a family of oxygenated C20 fatty acids (FAs) that can be grouped into prostaglandins, leukotrienes and epoxides (Smith, 1989). Prostaglandins, including prostaglandin E_2, are synthesised when arachidonic acid (AA) is liberated from glycerophospholipids by cytoplasmic phospholipase A_2 (cPLA$_2$) (Leslie, 2004; Murakami et al., 1997). Thereafter, AA is oxygenated by cyclooxygenases (COX) (DeWitt and Smith, 1988; Funk et al., 1991; Hemler and Lands, 1976; Yokoyama et al., 1988) and converted to various compounds by one of several enzymes including, PGE_2 synthases (Dennis and Norris, 2015) (**Figure 3. 1**). Several studies have shown that enzymes of the eicosanoid biosynthesis pathway localize with lipid droplets (LDs) (Accioly et al., 2008a; Bandeira-Melo et al., 2011; Bozza and Viola, 2010). Studies also indicate that an increase in LD numbers lead to an enhanced capacity for eicosanoid production. When human dermal microvascular endothelial cells were exposed to tumour necrosis factor α (a pro-inflammatory cytokine), lipid droplets with unsaturated lipids were formed in a time dependent manner (Czamara et al., 2017). These unsaturated lipids in the LDs also contained AA, which implies a role related to the eicosanoid synthesis pathway.
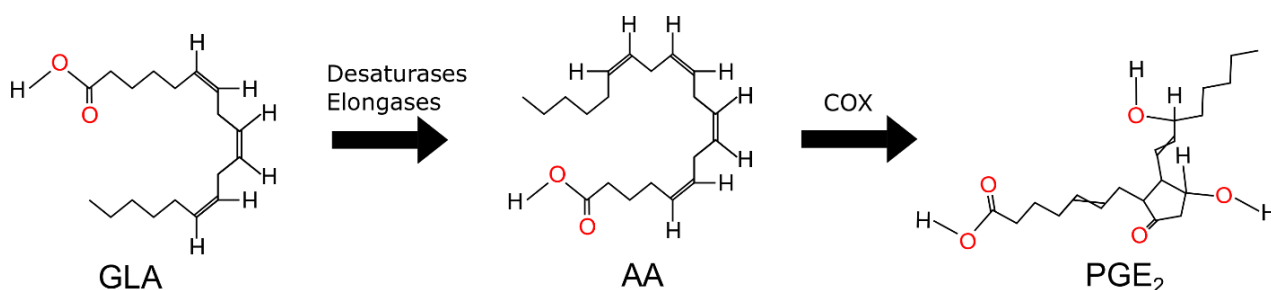
Figure 3. 1. PGE$_2$ biosynthesis. Biosynthesis of PGE$_2$ begins when γ-linolenic acid (GLA) is converted to arachidonic acid (AA) by elongases and desaturases. Thereafter, AA is converted to PGE$_2$ by cyclooxygenases (COX).

In immunity, eicosanoids regulate cytokine production, antibody formation and differentiation, cell proliferation and migration and antigen presentation. When eicosanoids are over expressed during infections, they may be anti-inflammatory or modulate the adaptive immune system. High levels of prostaglandin E_2 (PGE$_2$) may divert the T helper cell class 1(Th1) response to a Th2 response (Shibata et al., 2005). Infections caused by parasites (Abdalla et al., 2008), fungi (Noverr et al., 2001) and intracellular bacteria (Rangel Moreno et al., 2002) have shown that high levels of PGE$_2$ has an immune-repressive effect and switches the immune response to Th2 (Shibata et al., 2005) which is less effective against intracellular parasites. The modulatory effects of eicosanoids in some important viral infections are

reviewed by Sander and co-workers (2017). The wide range of effects from increased viral replication, roles in pathogenesis to the inhibition of immune responses were reviewed.

In the previous chapter, supplementation with (γ-linolenic acid) GLA resulted in an increase in AA, which, as described above, is a substrate for PGE_2, while also increasing viral replication. This chapter aims to determine what effect saturated and unsaturated FA supplementation and subsequent infection with RV will have on PGE_2 production in monkey kidney (MA104) cells.

3.2. Materials and Methods

3.2.1. Mammalian cells, culture conditions and fatty acids

Monkey kidney (MA104) cells were cultured in complete growth media consisting of Dulbecco's Modified Eagle's medium (DMEM) with 2 % FBS, penicillin (10 000 U/ml), streptomycin (10 000 µg/ml), amphotericin B (0.25 µg/ml) and 1 % non-essential amino acids (NEAA) as described in chapter 2 section 2.2.1. MA104 cells were supplemented with one of the following 50 µM stearic acid (SA) (Sigma-Aldrich, USA), GLA (Sigma-Aldrich, USA), oleic acid (OA) (Sigma-Aldrich, USA) for 24 h (Tanaka et al., 2007) as described in chapter 2 section 2.2.2.

3.2.2. ELISA detection of PGE_2 levels

MA104 cells were seeded (~0.05 x 10^6 cells/ml) into 24-well culture plates, and grown to 70 – 80 % confluence in complete growth medium, where after medium was changed to complete growth media supplemented with 50 µM FA for 24 h (Tanaka et al., 2001b) as described in section 2.2.2 . SA11 was activated by incubation in serum-free DMEM with 10 µg/ml trypsin IX in for 30 min at 37°C. Following, FA supplementation, cells were infected with activated SA11 (the prototype rotavirus (RV) strain) at a multiplicity of infection (MOI) of 1, for 45 min in infection media [serum-free DMEM containing antibiotic mixture [penicillin (10 000 U/ml), streptomycin (10 000 µg/ml) and amphotericin B (0.25 µg/ml)] (Lonza, Switzerland), 1 % NEAA (Lonza, Switzerland) and 1 µg/ml porcine pancreas trypsin (Sigma-Aldrich, USA)]. After infection, the cells were incubated for 15 h in infection media: serum-free DMEM containing, antibiotic mixture, 1 % NEAA and 1 µg/ml trypsin IX (Sigma-Aldrich, USA). Uninfected and unsupplemented cells control as well as supplemented uninfected cells were processed in parallel under the same conditions. The production of PGE_2 was determined with a Prostaglandin E_2 EIA kit from Cayman Chemical Co. (USA). The assay relies on the competition between PGE_2 and PGE_2-acetylcholinesterase (tracer) for a limited amount of PGE_2 monoclonal antibody. As the tracer is kept constant while the concentration of PGE_2 varies, the amount of tracer that binds to the PGE_2 monoclonal antibody will be inversely proportional to the amount of PGE_2. The concentrations (pg/ml) of PGE_2 in the samples were

calculated according to the reference calibration curves of standards (PGE$_2$, supplied with the kit). ELISA was carried out on three biological replicates, at two different dilutions, each in triplicate. To ensure that RV yield was not due to variation in cell number before infection, supplemented cells were counted to ensure that normalization could be achieved. Briefly, supplemented cells were trypsinized (Lonza, Switzerland) for 5 min to detached cells from the 25 cm^2 cell culture flask. Cells were harvested by centrifugation 800 x g for 5 min. The cells were resuspended in 1 ml DMEM medium and 100 µl of this suspension was added to 100 µl Trypan Blue and thoroughly mixed. Viable cells were counted and the number of cells were determine using a cell-counting chamber (Neubauer) and inverted light microscope (Nikon Eclipse TS100).

3.2.3. Authentication of prostaglandin E$_2$ produced by MA104 cells using LC-MS/MS

In order to confirm authentic PGE$_2$ production, MA104 cells were seeded (~0.3 x 10^6 cells/ml) in 6-well culture plates, supplemented with 50 µM GLA for 24 h and subsequently infected with SA11 at a multiplicity of infection (MOI) of 0.1 and 1 for 16 h. Supernatants from cell culture wells were collected. For each analysis, a 500 µl aliquot was spiked with 10 µl of PGE$_2$ (100 ng/ml each) as internal standards. Citric acid (Sigma-Aldrich, USA) and butylated hydroxytoluene (Sigma-Aldrich, USA) was added to a final concentration of 80 mM and 0.1 %, respectively, to prevent free radical catalysed lipid peroxidation. Prostaglandin E$_2$ and PGD$_2$ were extracted by adding 2 ml hexane/ethyl acetate (1:1, v/v) followed by vortex mixing for 1 min. After centrifugation for 5 min at 1000 x g at 4 °C, the upper organic phase was removed. The extraction procedure (Caoa et al., 2008) was repeated twice more, the organic phases combined and evaporated to dryness under a stream of nitrogen gas.

Twenty microliters of each sample was separated on a C18 column (Luna 3um C18, 150x3mm, Phenomenex) at a flow rate of 200 ul/min using 0.1 % formic acid (mobile phase A) and acetonitrile with 0.1 % formic acid (mobile phase B). The column was equilibrated and loaded at 20 % of mobile phase B, increasing to 42.5 % B over 50 minutes, 95 % B for 10 minutes, followed by re-equilibration at 20 % B for a total run time of 70 minutes. The targeted analysis for PGE$_2$ used 2 multiple reaction monitoring transitions. 351.1 > 315.2 (quantifier) and 351.1 > 271.2 (qualifier) in negative ionisation mode. Ionspray voltage was set at 4500V, nebuliser gas (GS1) was at 40 psi and heater gas (GS2) at 30 psi with the heater temperature set at 400 °C.

Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing were performed using Analyst 1.5 (AB SCIEX) software. To analyse the sample a targeted Multiple Reaction

Monitoring (MRM) workflow was followed on the instrument. All compound and source dependent parameters were optimised using compound optimization in Analyst 1.5.2. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions (if available) were used as qualifiers. The qualifier serves as an additional level of confirmation for the presence of the analyte, the retention time for these transitions needs to be the same.

3.2.4. Co-localization studies for lipid droplets, PGE$_2$ and rotavirus viroplasm

3.2.4.1. NSP2 expression and antibody production

Escherichia coli BL21 cells previously transformed with a recombinant pET28a vector, containing the SA11 NSP2 ORF in-frame with a 6x His-tag sequence at the 3' end of the ORF (previously prepared by Fenja Gerpott, DAAD Intern), was inoculated in lysogeny broth media (Bertani, 1951) containing 100 µg/ml of kanamycin (Sigma-Aldrich, USA). After 3 h, isopropyl β-D-1-thiogalactopyranoside (Thermo Fischer Scientific, USA) was added at a final concentration of 0.4 mM to induce the expression of NPS2. The cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C. The pellet was suspended in sample buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0), and cells were lysed with a One Shot Cell Disruptor (Constant Systems, Ltd.) at 30 kpsi. After disruption, the supernatant was ultracentrifuged at 100 000 x g for 2 h. NPS2 was purified using Ni-NTA Superflow Columns (Qiagen, Germany). Briefly, Ni-NTA Superflow Columns were calibrated by adding 10 ml Buffer NPI-10 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10mM imidazole, 0.05 % Tween® 20, pH 8) where-after lysates were loaded onto columns. Columns were then washed twice by adding 10 ml of Buffer NPI-20 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20mM imidazole, 0.05 % Tween® 20, pH 8) , followed by the elution of NPS2 in 3 ml Buffer NPI-250 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250mM imidazole, 0.05 % Tween® 20, pH 8). The purification was evaluated by using a 10 % SDS-PAGE gel according to Laemmli, (1970) in the presence of the PageRuler™ prestained protein ladder (Thermo Fisher Scientific, USA). The gel was stained with Coomasie Blue R250 using the Fairbanks method (Fairbanks et al., 1971; Wong et al., 2000). The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used to determine the protein concentration. The purified NSP2 protein was submitted to Deltamune (Pty.) Ltd (Pretoria) to raise polyclonal antibodies in rabbits (*Oryctolagus cuniculus*).

3.2.4.2. Acetylsalicylic acid toxicity assay

Acetylsalicylic acid (aspirin), an irreversible, non-specific inhibitor of cyclooxygenases (COX), is required by the Eicosacell protocol as a negative control. To determine the concentration at which acetylsalicylic acid is non-toxic to the MA104 cells, a toxicity assay was carried out. MA104 cells were seeded (~0.05 x 10^6 cells/ml) onto coverslips in 24 well plates and grown

to 70 – 80 % confluence. Cells were treated with several concentrations of acetylsalicylic acid (from 0 µM to 5 mM) and incubated for 3 h at 37 °C and 5 % CO_2. Following acetylsalicylic acid treatment, the cell viability of MA104 cells was determined as described in section 3.2.2.

3.2.4.3.   Microscopic localization of LDs, PGE_2 and NSP2

MA104 cells were seeded (~0.05 x 10^6 cells/ml) onto coverslips in 24-well culture plates and cultured to 70 – 80 % confluence in complete growth media. Cells were supplemented with 50 µM of each FA and incubated overnight at 37 °C with 5 % CO_2. Following FA supplementation, cells were infected with SA11 at a MOI of 5 for 45 min infection media. After infection, the cells were washed three times with phosphate buffer saline (PBS) (Sigma-Aldrich), incubated for 2 h in infection media. Various controls were included: uninfected and unsupplemented cells, supplemented, but uninfected cells and cells treated with 1 mM acetylsalicylic acid (see section 3.2.4.3) were processed in parallel under the same conditions. Localization of PGE_2 was performed by the EicosaCell method (Bandeira-Melo et al., 2011). Briefly, cells were incubated in 0.5 % 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Sigma-Aldrich, USA) in PBS for 1 h at 37 ℃ and washed (3x) with PBS. The cells were then incubated with the PGE_2 monoclonal antibody (Cayman chemical, USA) and RV NSP2 polyclonal antibody (Deltamune (Pty.) Ltd (Pretoria)) with 10 % Caprine serum (provided by Dr. Floris Coetzee, Dan Pienaar Veterinary Clinic, Bloemfontein) for 1h at 37 ℃ followed by washing (3x) with PBS. Cells were then incubated with Alexa Fluor™ 594 goat anti-mouse IgG (H+L) (Thermo Fischer Scientific, USA), Alexa Fluor 532™ goat anti-rabbit IgG (H+L) secondary antibody and 1 µM BODIPY® 493/503 stain (Thermo Fischer Scientific, USA) for 1 h at room temperature in dark. Cells were then washed three times for at least 5 min each and then coverslips were placed on slides. Analyses were performed using confocal laser scanning microscopy (CLSM) [Carl Zeiss Confocal LSM 780 Elyra S1 (Oberkochen, Germany)]. Images were standardized by keeping settings for all images universal. This includes the laser intensity, the pinhole size and the gain of the detector. Three confocal images, each containing several M104 cells, were taken to ensure that representative results were obtained.

3.2.5. Statistical analysis

All experiments were performed at least in triplicate unless otherwise stated. In cases where three or more values were obtained, the ANOVA test (Fisher, 1925) and the Tukey-Kramer (Tukey, 1949)ad hoc test were performed to determine the significance of the data sets unless otherwise stated. A p value of 0.05 or less was considered significant.

3.2.6. Ethics

Ethical approval was obtained for the caprine serum from the Animal Research Ethics Committee at UFS with approval number: UFS-AED2017/0069 (Appendix A). Generation of

the anti-NSP2 rabbit polyclonal antibodies was outsourced to Deltamune (Pty.) Ltd (Pretoria) and raised at the company's Department of Agriculture, Forestry and Fisheries (DAFF) approved facilities. Approval for the use of the caprine serum and polyclonal antibodies in terms of Section 20 of the Animal Disease Act of 1984 was obtained from DAFF (Appendix A).

3.3.   Results

3.3.1.   *PGE$_2$ induction by rotavirus in supplemented MA104 cells*

To determine the amount of PGE$_2$ produced by supplemented and SA11 infected MA104 cells the Prostaglandin E$_2$ EIA kit for PGE$_2$ isomers were used. **Figure 3. 2** shows the eicosanoid concentration (pg/ml) obtained. The number of cells after supplementation, but prior to infection within each sample, did not differ significantly and thus no normalisation was required. It can be clearly seen that PGE$_2$ is produced during physiological conditions by MA104 cells, with the uninfected control producing (104.02 [±7.63]) pg/mL PGE$_2$. Infection of MA104 cells with SA11 leads to a significant increase in the PGE$_2$ produced (144.83 [±9.41]) pg/mL when compared to the uninfected, unsupplemented MA104 cells control. Supplementation of MA104 cells with albumin (89.45 [±7.10]) pg/mL, stearic acid (SA) (98.61 [±7.79]) pg/mL, oleic acid (OA) (97.61 [±6.36]) pg/mL had no significant effect on the production of PGE$_2$. GLA supplementation of uninfected MA104 cells significantly increased the amount of PGE$_2$ produced (168.92 [±15.27]) pg/mL. Furthermore, infection of supplemented MA104 cells with SA11 increased the amount of PGE$_2$ produced in cells supplemented with albumin (154.84 [±7.16]) pg/mL, SA (162.47 [±24.64]) pg/mL, OA (136.53 [±136.53]) pg/mL, to the same level as for unsupplemented, but infected cells. In contrast, supplementation of MA104 cells with GLA, prior to infection, increased (343.71 [±31.60]) pg/mL the amount of PGE$_2$ more than two-fold when compared to the unsupplemented infected cells.
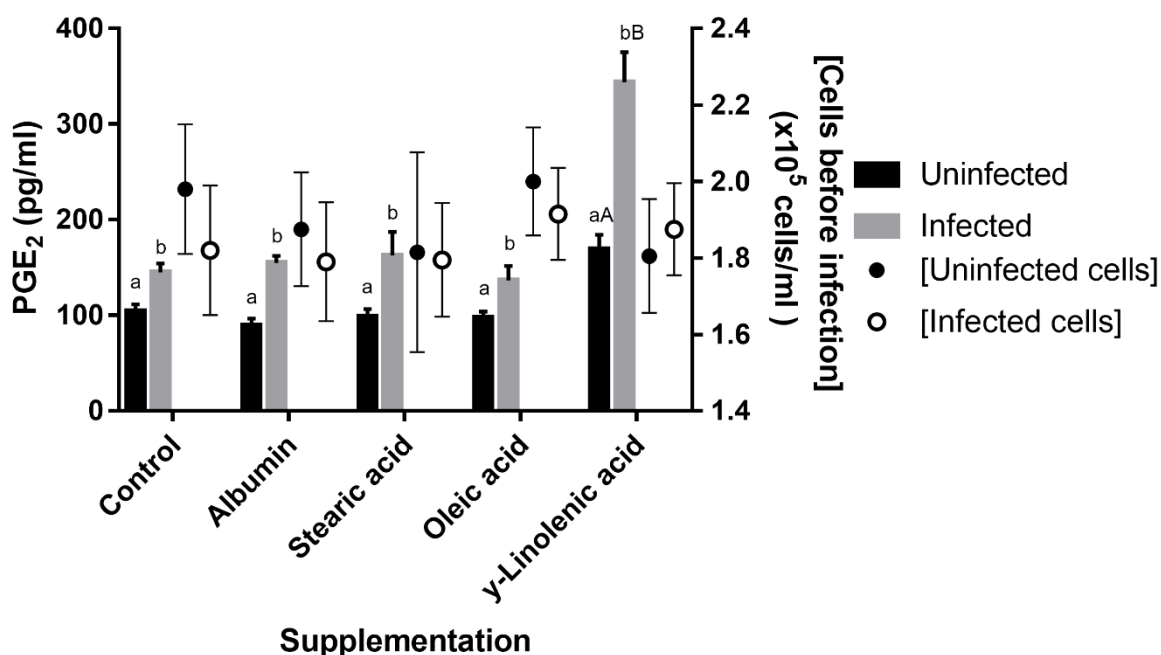
Figure 3. 2. PGE$_2$ induction by SA11 in supplemented MA104 cells. MA104 cells were supplemented with 50 µM of each respective FA for 24h, where after supplemented MA104 cells were infected for 16 h. ELISA was used to determine the concentration of PGE$_2$ 16 h post infection. Primary y-axis pertains to PGE2 concentration rate of replication, while the secondary y-axis pertains to cells concentration before infection. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars.

3.3.2. Authentication of PGE$_2$ production in MA104 cells

Production of PGE$_2$ was confirmed by LC-MS/MS (**Figure 3. 3**) with a PGE$_2$ isomer separation method to authenticate the production of PGE$_2$ (Brose et al., 2011). To authenticate the detected PGE$_2$, samples were spiked with PGE$_2$ to determine the retention time. As can be seen from the spiked sample (**Figure 3. 3A$_2$ and B$_2$**) two transitions of PGE$_2$ required for authentication were observed at a retention time of ~ 45 min. The corresponding peaks were also observed in the unspiked sample (retention time ~45 min) (Figure 3. 3A1 and A2) indicating that PGE$_2$ was produced by MA104 cells during SA11 infection. It is important to

note that the MA104 cells infected at a higher MOI resulted in a higher peak of PGE_2 detected, possibly indicating a dose-dependent increase in PGE_2 during SA11 infection.
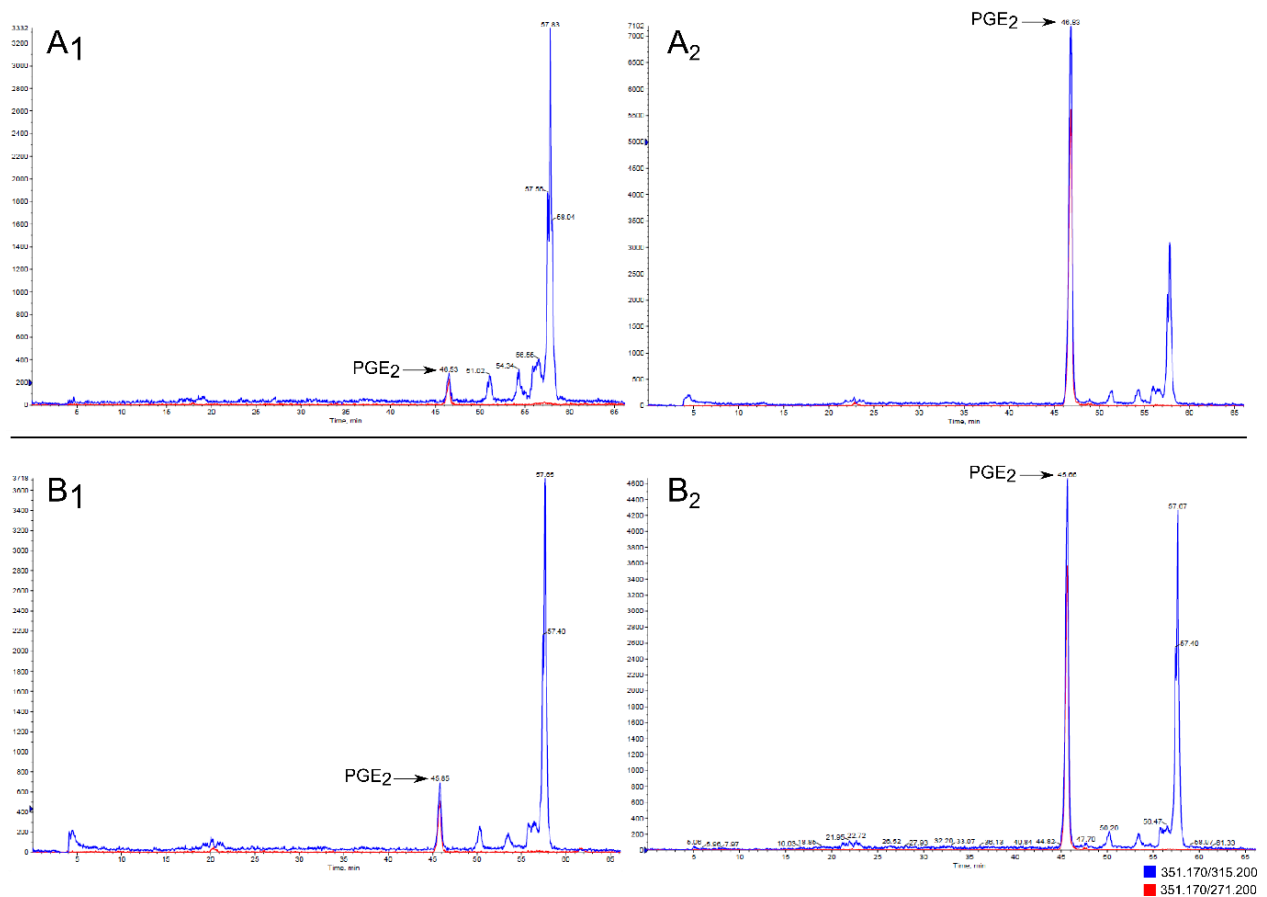
Figure 3. 3. Confirmation of extracellular prostaglandin E2 (PGE_2) production by GLA supplemented and SA11 infected MA104 cells by LC-MS/MS. MA104 cells were infected with SA11 at a MOI of 0.1 (A) and 1 (B) for 16 h. A_1 and B_1) Supernatant spiked with 10 ng of PGE_2 prior to analysis. A_2 and B_2) The PGE_2 of MA104 cells supplemented with GLA and infected with SA11. Profiles indicate transitions 351.1 > 315.2 (quantifier) and 351.1 > 271.2 (qualifier) of PGE_2 in each sample. Corresponding colours of transitions are indicated on the bottom of the figure. Arrows indicate PGE_2 peaks.

3.3.3. Confocal scanning laser microscopy of the co-localization of LDs, PGE_2 and NPS2 in supplemented and infected MA104 cells

3.3.3.1. NSP2 expression and antibody production

To confirm the co-localization of NSP2 with both LDs and PGE_2 within MA104 cells, a NSP2-specific antibody was required. Such an antibody is not commercially available. Using a recombinant expression vector containing the SA11-N5 NSP2 ORF (Mlera et al., 2013) and an in-frame His-tag, the NSP2 protein was successfully expressed containing a C-terminal His tag (**Figure 3. 4**). Purification via immobilised metal affinity chromatography of NSP2 was successful as a single band of approximately 35 kDa (Mason et al., 1983) was observed on a 10 % SDS-PAGE (**Figure 3. 4**, **lane 4**).
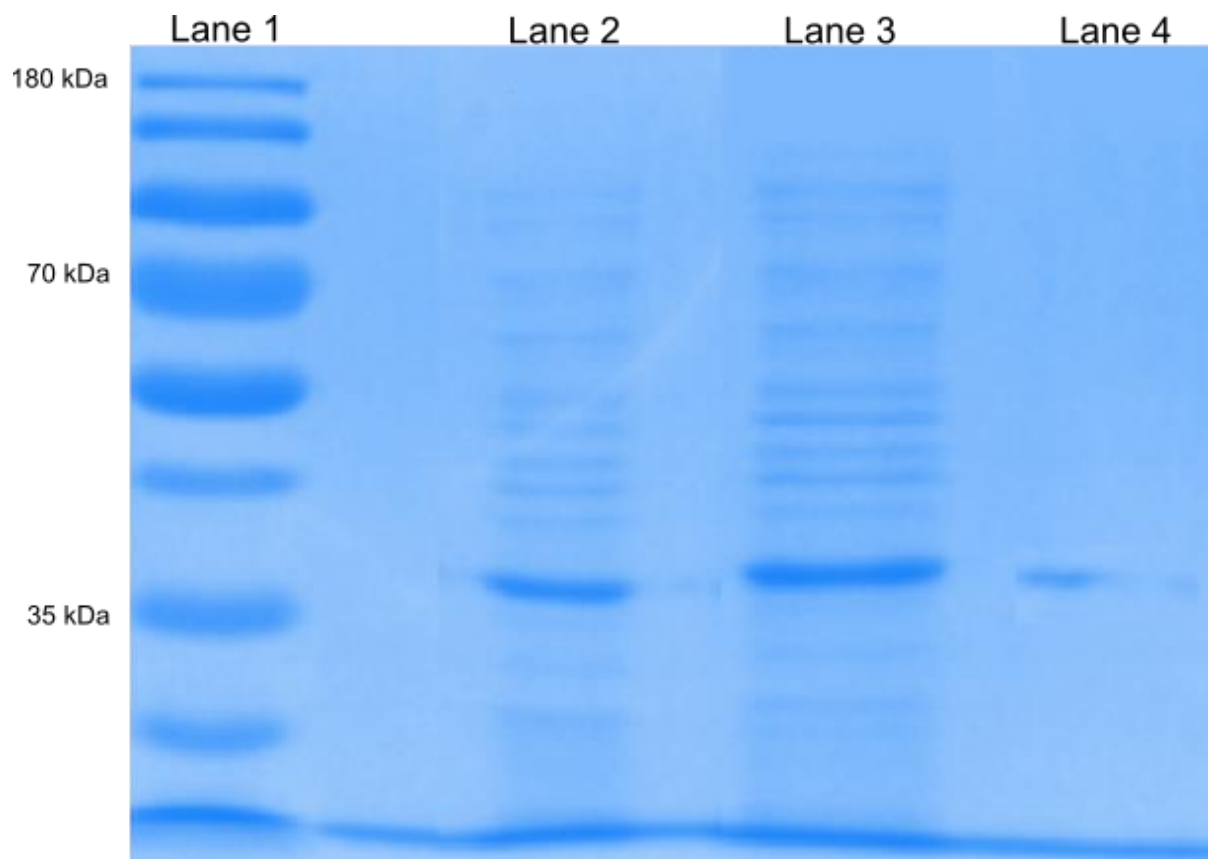
Figure 3. 4. SDS-PAGE of the expression and purification of NSP2. The gel was stained with Coomassie Blue R250 (Fairbanks et al., 1971; Wong et al., 2000) and the size marker was a PageRuler™ prestained protein ladder (Thermo Fisher Scientific, USA). Lane 1 includes the prestained PageRuler™ protein ladder, Lane 2 contains the lysate of the recombinant *E. coli* BL21 cells after disruption, Lane 3 and Lane 4 contains purified NSP2 after ultracentrifugation and NI-NTA columns, respectively.

3.3.3.2.   Acetylsalicylic acid cytotoxicity in MA104 cells

Concentrations of acetylsalicylic acid up to 1 mM appears to have no significant effect on the viability of MA104 cells after 3 hours of incubation. In contrast, concentrations of acetylsalicylic acid above 2.5 mM appear to significantly decrease the viability of MA104 cells, with 2.5 mM of acetylsalicylic acid decreasing the number of viable cells 2.6 fold while 5 mM of acetylsalicylic acid kills all the MA104 cells (**Figure 3. 5**).
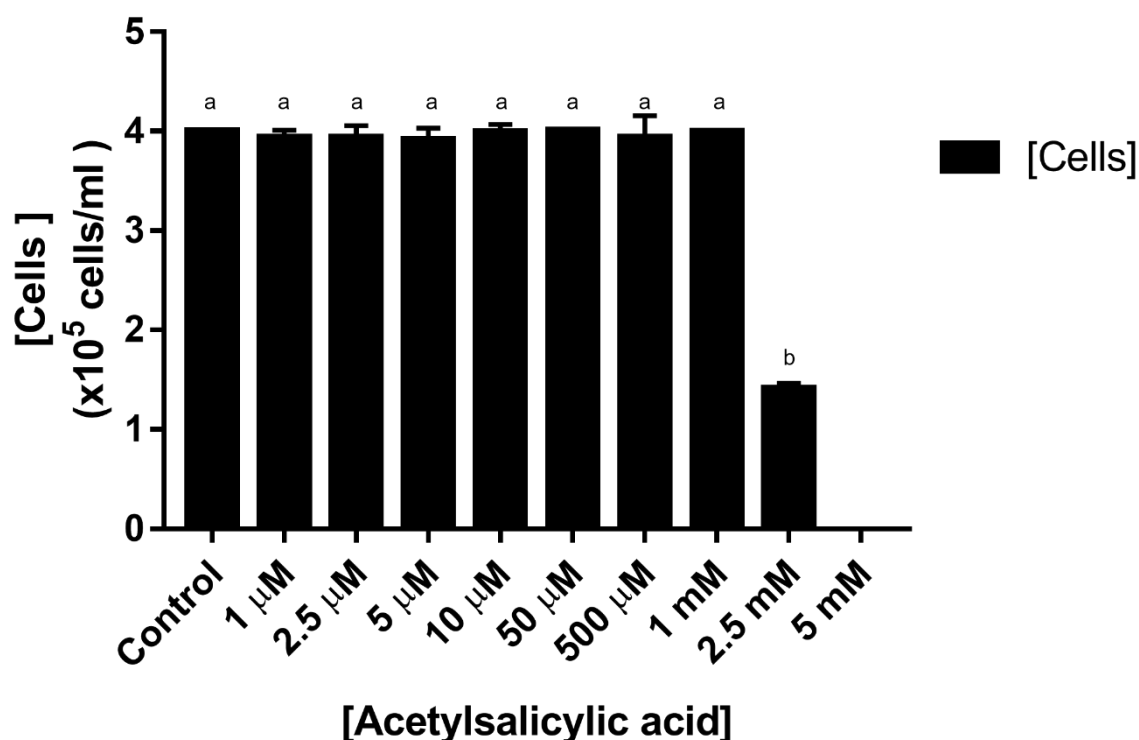
Figure 3. 5. The toxicity of acetylsalicylic acid on MA104 cells. MA104 cells were subjected to different concentrations of acetylsalicylic acid. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars. Letters indicate significant differences between tested conditions.

3.3.3.3.   .Microscopic localization of LDs, PGE$_2$ and NSP2

To ensure that fluorescence is not due to non-specific binding of the secondary fluorescent-conjugated antibodies, negative controls were included (**Figure 3. 6**). The absence of primary antibodies to NSP2 and PGE$_2$ as well as the absence of BODIPY 493/503 yielded no fluorescence, indicating that the secondary fluorescent-conjugated antibodies are specific for their respective primary targets (**Figure 3. 6A**). Additionally, a negative control which does not make use of EDAC for cross-linking of PGE$_2$ was required (**Figure 3. 6B**). The absence of EDAC also leads to an absence of fluorescence.
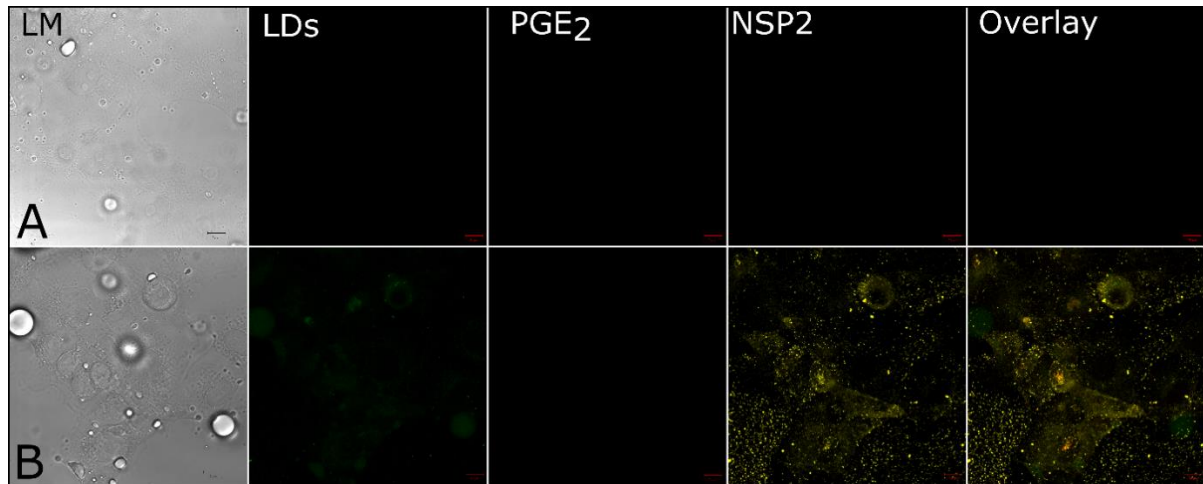
Figure 3. 6. Negative controls for confocal laser scanning microscopy. Confocal images of MA104 cells co-stained with BODIPY 483/503, antibodies to NSP2 and PGE_2. The visualisation of LDs, PGE_2 and NSP2 (A) without the presence of primary antibodies and BODIPY 493/503 and (B) non-EDAC treated cells. Light microscopy (LM) images show MA104 morphology, BODIPY 493/503 visualises LDs, Alexa Fluor™ 594 visualise PGE_2 and Alexa Fluor 532™ visualises NSP2. Overlay shown in the final panel. Scale bar, 10 μm.

Using confocal microscopy and MA104 cells, we were able to minimally visualise both LDs and PGE_2 (**Figure 3. 7A**). Furthermore, the use of acetylsalicylic acid to inhibit the production of PGE_2 appears to only have a minimal effect, when comparing panel A with panel B in **Figure 3. 7**. This is due to the successful visualisation of PGE_2 in cells treated with acetylsalicylic acid (**Figure 3. 7B**). It should however be noted that the staining of LDs with BODIPY 493/503 caused several problems which will be discussed later.
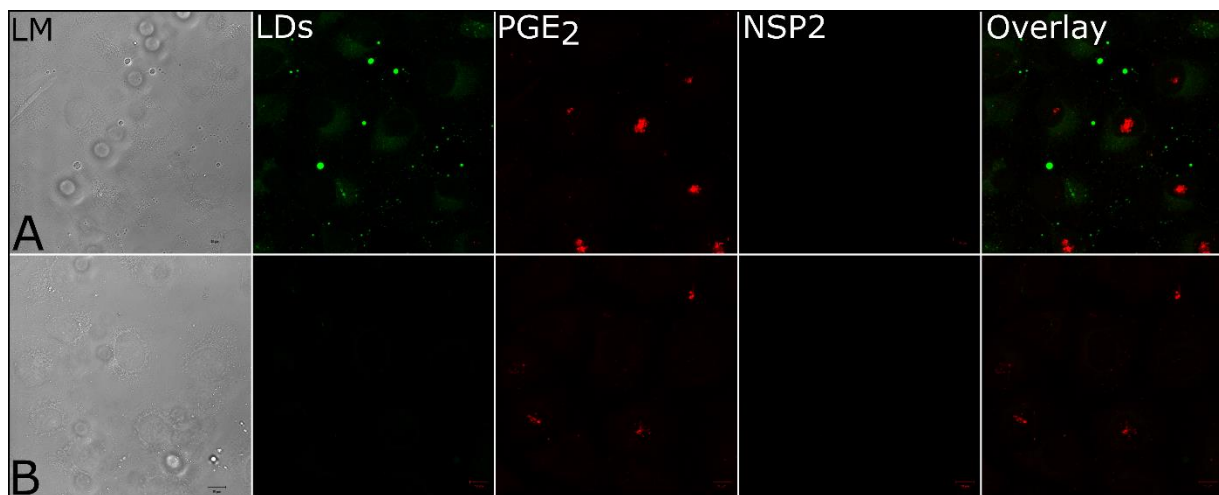
Figure 3. 7. The effect of acetylsalicylic acid on the visualisation of PGE_2 in uninfected MA104 cells. Confocal images of uninfected MA104 cells co-stained with BODIPY 483/503, antibodies to NSP2 and PGE_2. The visualisation of LDs, PGE_2 and NSP2 in the (A) absence and (B) presence of 1 mM acetylsalicylic acid. Light microscopy (LM) images show MA104 morphology, BODIPY 493/503 visualises LDs, Alexa Fluor™ 594 visualise PGE_2 and Alexa Fluor 532™ visualises NSP2. Overlay shown in the final panel. Scale bar, 10 μm.

The co-staining of LDs, PGE$_2$ and NSP2 in SA11-infected cells, showed co-localization between LDs, NSP2 and PGE$_2$ (**Figure 3. 8**). Although, co-localization was seen between these three components it did not occur the majority of the time (**Figure 3. 8**A). It should be noted that there appears to be a relative increase in the amount of red fluorescence when comparing the infected (**Figure 3. 8**) to the uninfected controls (**Figure 3. 7**). In several instances, there was co-localization between at least two of the components. The use of acetylsalicylic acid appears to have minimal effect on the production of PGE$_2$ or on viroplasm formation as fluorescence from PGE$_2$ (red) and NSP2 (yellow) appears to have the same relative amount of fluorescence when comparing SA11 infection of MA104 cells in the presence and absence of acetylsalicylic acid.
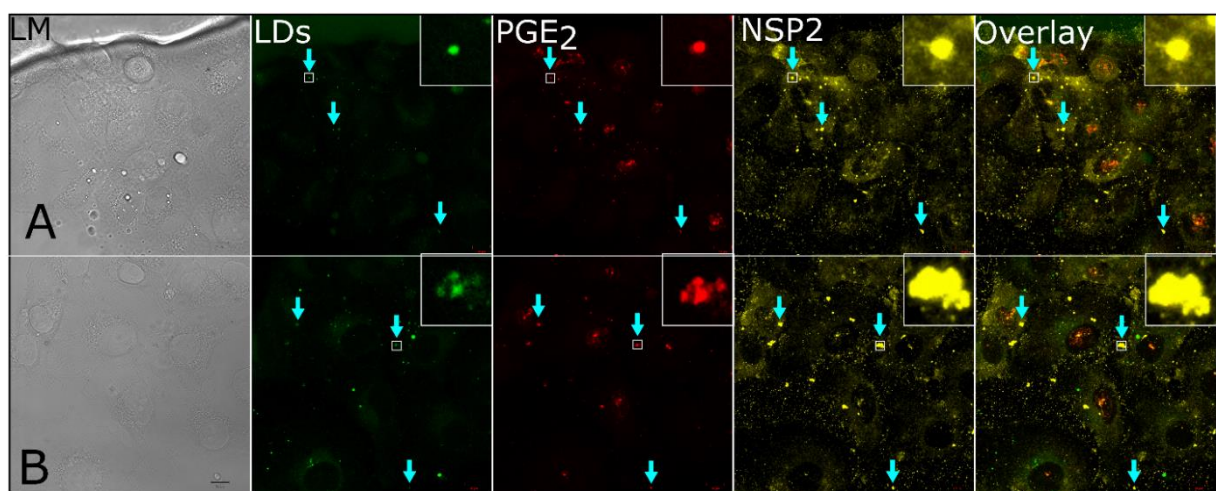
Figure 3. 8 Confocal images of MA104 cells co-stained with BODIPY 483/503, antibodies to NSP2 and PGE$_2$. The co-localization of LDs, PGE2 and NSP2 in the (A) absence and (B) presence of 1 mM acetylsalicylic acid. Light microscopy (LM) images show MA104 morphology, BODIPY 493/503 visualises LDs, Alexa Fluor™ 594 visualise PGE2 and Alexa Fluor 532™ visualises NSP2. Overlay shown in the final panel. Scale bar, 10 µm. Blue arrows indicate where all three components co-localise.

Finally, confocal microscopy (CM) data was generated to determine the effect of GLA supplementation on PGE$_2$ production and SA11 replication (**Figure 3. 9**). Acetylsalicylic acid appeared to have minimal effect on the production of PGE$_2$, although there appears to be a relative decrease in the fluorescence of NSP2 (yellow) when comparing SA11-infected MA104 cells in the presence or absence of acetylsalicylic acid. While the data does not conclusively indicated that PGE$_2$ and LDs co-localise, due to technical issues (discussed later), co-localization between PGE$_2$ and NSP2 is observed, as can been seen by the lack of red (**Figure 3. 9**) and the presence of orange (**Figure 3. 9**). It should be noted that LDs (Fujimoto and Ohsaki, 2006a), PGE$_2$ (Accioly et al., 2008b; Park et al., 2006) and NSP2 (Kattoura et al., 1994) were all located within the cytoplasm as expected.
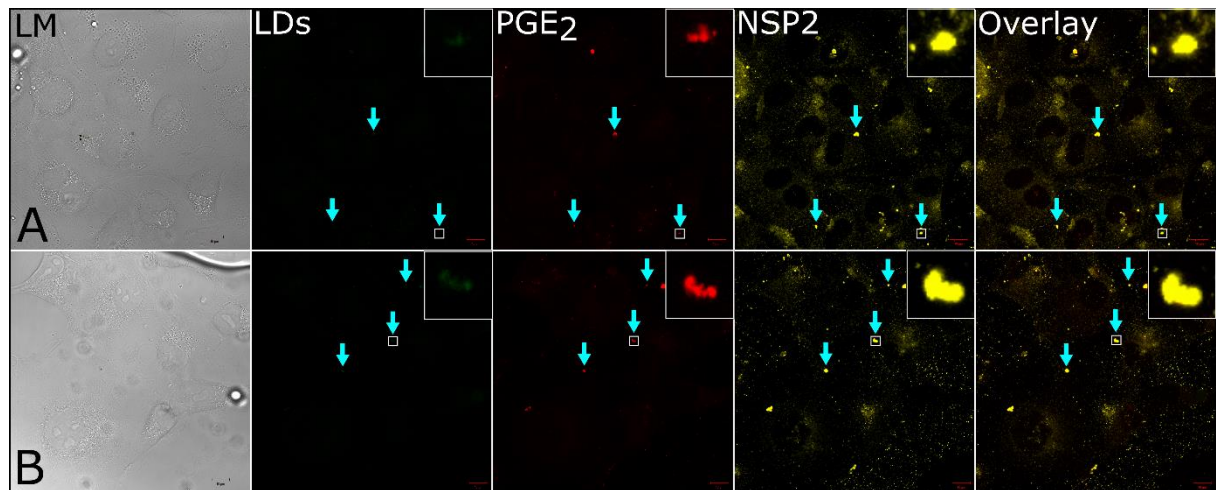
Figure 3. 9 The effect of acetylsalicylic acid on the co-localization of rotavirus NPS2 with PGE₂ in GLA supplemented, infected MA104 cells. Confocal images of GLA supplemented and infected MA104 cells co-stained with BODIPY 483/503, antibodies to NSP2 and PGE₂. The co-localization of LDs, PGE₂ and NSP2 are visualised in the (A) absence and (B) presence of 1 mM acetylsalicylic acid. Light microscopy (LM) images show MA104 morphology, BODIPY 493/503 visualises LDs, Alexa Fluor™ 594 visualise PGE₂ and Alexa Fluor 532™ visualises NSP2. Overlay shown in the final panel. Scale bar, 10 μm. Blue arrows indicate co-localization between LDs, NSP2 and PGE₂.

3.4. Discussion

Eicosanoids are signalling molecules that are produced from the oxidation of AA or other poly-unsaturated FAs, and are known for their roles in immunity (Esser-von Bieren, 2017). They are synthesised after activation by mechanical trauma, bacterial peptides, allergens or cytokines and growth factors (Bozza et al., 2011). Of particular importance for is the role that PGE₂ has on a wide range of viral infections (Sander et al., 2017). Here we show by ELISA and confocal microscopy that the prototype rotavirus strain, SA11, is capable of increasing PGE₂ during *in vitro* RV replication.

Several enzymes involved in the production of eicosanoids, those from AA, have been found to associate with LDs. Cytosolic PLA₂ (cPLA₂) has been found to predominately localise to the surface of LDs rather than within the core (Moreira et al., 2009; Wenguiyu et al., 1998; Wooten et al., 2008). Interestingly, the translocation of cPLA₂ to cellular membranes is highly regulated by intracellular levels of calcium (Nalefski et al., 1994), which can be increased during RV infection by NSP4 (Hyser et al., 2010). RV infection could thus increase the rate of cPLA₂ translocation, subsequently increasing the amount of AA liberated from phospholipids in the membrane. In contrast, enzymes such as COX, have been found to localize within the core of LDs (Accioly et al., 2008a; Bozza et al., 1997; D'Avila et al., 2006; Dvorak et al., 1992, 1993). More downstream enzymes, like PGE₂-synthase, have been shown to localise within LDs (Accioly et al., 2008a; Araújo-Santos et al., 2010; Bozza et al., 1997, 2009) , although it should be noted that it is still unclear how these integral membrane spanning proteins are

accommodated within the phospholipid monolayer or how they are targeted to LDs (Bozza et al., 2011). In addition, PGE_2 has been shown to co-localise with adipose differentiation-related protein (ADRP), which is a known LD-associated protein (Brasaemle et al., 1997; Miura et al., 2002) in several cells lines, such as macrophages and CACO-2 cells (Bandeira-Melo et al., 2011). Endothelial and epithelial cells involved in various pathological conditions, have been shown to contain an increased number of LDs associated with PGE_2 production (Accioly et al., 2008a; Dvorak et al., 1992; Dvoràk et al., 1994; Plotkowski et al., 2008; Scarfo et al., 2001).

By staining LDs with BODIPY as well as PGE_2 and NSP2 with fluorescent-conjugated secondary antibodies, we showed co-localization between the various components. The data shows that there is minimal co-localization of LDs with either PGE_2 or NSP2. This is partly in concurrence with studies that have found that RV proteins co-localise with LDs (Cheung et al., 2010; Crawford and Desselberger, 2016; Gaunt et al., 2013b), while other studies have shown that PGE_2 and PGE_2-biosynthetic enzymes also co-localise with LDs (Accioly et al., 2008a; Bozza et al., 2011; Bozza and Viola, 2010; Silva et al., 2009). Of importance is the novel data that shows a strong co-localization between NSP2 and PGE_2. This indicates a possible role for viroplasms in increasing PGE_2 production and subsequent enhancing role for PGE_2 in the RV replication cycle, as has been found for several other viral replication cycles (Sander et al., 2017). Lastly, although there appears to be some co-localization of all three components, it was not much of the time, which could once again be due to technical difficulties.

It should, however, be noted that several problems were encountered during the visualisation of LDs, PGE_2 and NSP2. Firstly, due to the unavailability of a confocal microscope that could distinguish between green (493/503 nm), yellow (532/553 nm) and red (590/617 nm), samples had to be shipped for analysis, which was suboptimal and introduced air bubble formation. In addition, this also prevented the optimisation of optimal fluorescence for each fluorophore. Secondly, due to the non-specific nature of BODIPY 493/503 binding to all neutral lipids, bleaching of green fluorescence occurred in several samples. The use of antibodies that are specific for proteins known to co-localise with LDs, such as perilipin A (Blanchette-Mackie et al., 1995) or adipose differentiation-related protein (Brasaemle et al., 1997), could prevent this. Furthermore, due to the close emission spectrum of the fluorophores, lambda scans were required to distinguish between their emissions wavelengths. This led to a significant decrease in the fluorescence obtained for BODIPY 493/503. Either the green or yellow fluorophore can be replaced with a blue (350 nm) fluorophore which will also allow for optimisation. This resulted in the inability to quantitatively determine the concentration of PGE_2 as well as NSP2 within LDs. Thirdly, the addition of acetylsalicylic acid, an irreversible and non-specific inhibitor of COX (Vane, 1971), appeared to have minimal effect on the production of PGE_2. This failure

could be due to the time of addition (3 h before fixation) compared to the addition of acetylsalicylic acid 1 h before fixation in the EicosaCell protocol (Bandeira-Melo et al., 2011). We found that acetylsalicylic acid concentration below 1 mM had no significant effect on the viability of MA104 cells, while acetylsalicylic acid concentrations of 2,5 mM and 5 mM significantly decreased the viability of MA104 cells. The concentration of toxicity is consistent with what is known in literature, where cell lines such as HEK-293 and HT-29 show acetylsalicylic acid toxicity at concentrations above 0.5 mM (Massimi et al., 2015; Pathi et al., 2012; Subbegowda and Frommel, 1998).

The ELISA results showed that SA11 could increase the production of PGE_2 irrespective of supplementation, indicating some role for PGE_2 within SA11 replication. During RV infection of malnourished pigs, it was found that malnourished pigs had elevated levels of PGE_2 and this corresponded to a longer duration of diarrhoea (Zijlstra et al., 1999). Furthermore, in the late 1980s it was found that children infected with RV had significantly higher levels of PGE_2 within plasma and stool when compared to healthy children (Yamashiro et al., 1989). Giving acetylsalicylic acid to RV infected individuals caused diarrhoea to cease earlier (Yamashiro et al., 1989), and subsequent studies showed that inhibition of COX by indomethacin reduced RV infection by 85 to 50 % (Rossen et al., 2004). MA104 cells supplemented with GLA and then subsequently infected with SA11 increased the amount of PGE_2 almost 4-fold compared to unsupplemented and infected MA104 cells, further indicating a role for PGE_2. As with cell lines like ewe endometrial and HEP-G2 cells and rats (Quoc and Pascaud, 1996) supplemented with GLA (Cheng et al., 2004; Sergeant et al., 2016; Viau et al., 2012), the supplementation of MA104 cells with GLA showed a hyper-production of PGE_2. Furthermore, the infection of MA104 cells by SA11 at two difference MOIs showed that the production of PGE_2 could be dose-dependent. Taken together the results indicate that during RV infection, PGE_2 production is increased in MA104 cells, and that supplementation with GLA of these infected cells can increase the amount of PGE_2 produced.

The importance of pathogens' ability to modulate the fatty acid (FA) composition of host cells for their own replication is increasingly being investigated. This is due to the downstream metabolites of FAs that are critical in determining the strength and type of the immune response. This study sought to identify the effects that FA saturation has on rotavirus replication. The specific objectives were: (i) to investigate what effect the change in cellular FA composition (neutral lipids and phospholipids), due to the supplementation of FAs at different degrees of saturation, may have on the yield of infectious rotavirus; and (ii) to investigate the effect of the modulation of FA composition on rotavirus replication and subsequent effects on eicosanoid production.

The supplementation of MA104 cells with each respective FA changed the lipid profile of both the neutral and phospholipid fraction in unique ways. The supplementation of unsaturated FAs, (oleic acid (OA: 18:1) and γ-linolenic acid (GLA: 18:3), had a more pronounced effect compared to the saturated FA, (stearic acid (SA: 18:0). As expected, supplementation with OA increased the relative percentage of OA, while GLA supplementation increased the percentage of GLA as well as arachidonic acid (AA). Furthermore, the supplementation of MA104 cells with GLA (omega-6) increased the omega-6/omega-3 ratio, within both the neutral and phospholipid fractions while OA (omega-9) supplementation decreased the ratio within the neutral lipid fraction. The study affirmed that rotavirus is capable of modulating the lipid composition of supplemented MA104 cells (Gaunt et al., 2013b). Irrespective of supplementation, RV was capable to decrease the relative amount of OA and GLA within the neutral lipid and phospholipid fraction, respectively. Infection of supplemented cells also led to decreases in the omega-6/omega-3 ratio in cells supplemented with SA (neutral lipid), OA (neutral and phospholipids) and GLA (neutral and phospholipids). An important finding of this study is that supplementation of MA104 cells with unsaturated fatty acids increases the replication rate of rotavirus SA11. No significant change in rate of replication was observed during supplementation with the saturated fatty acid, SA. This is in contrast to Superti and co-workers (1995), who found that saturated fatty acids had an effect on the replication rate of RV. It should, however, be noted that they did not include SA, and dissolved their FAs in ethanol and not in a water-albumin solution. Supplementation with GLA increased the viral yield of SA11 by approximately 3-fold, while supplementation with OA increased the viral yield of SA11 by approximately 1.5-fold.

In concurrence with previous data (Yamashiro et al., 1989; Zijlstra et al., 1999), it was shown that RV is capable of increasing the amount of PGE_2 produced, irrespective of supplementation with FAs. However, it should be noted that GLA supplementation led to an

almost 2-fold increase in PGE_2. LC-MS/MS was employed to authenticate the production of PGE_2 during RV infection of MA104 cells. In addition, LC-MS/MS data indicated that the production of PGE_2 may be viral dose-dependent due to the difference in peak height for PGE_2 between a MOI of 0.1 and 1 for SA11. The production of PGE_2 during supplementation was co-visualised with LDs and NSP2, a protein that forms part of the viroplasm (Fabbretti et al., 1999), by CLSM. In addition, supplementation with GLA increased the amount of PGE_2 visualised. For the first time we were able to show the co-localization between NSP2 and PGE_2 and while partial co-localization between LDs and PGE_2 (Bozza et al., 2011) partial LDs and NSP2 (Cheung et al., 2010) was observed.

Taken together the data indicates that both OA and GLA supplementation can increase the rate of replication for SA11 within MA104 cells. This increase in replication could possibly occur by two methods. Firstly, supplementation of MA104 cells with GLA and OA seems to increase the number of LDs visualised (Rohwedder et al., 2014), which can subsequently increase the number of viral factories (viroplasms) formed during RV replication and subsequently increase the yield of virus (Cheung et al., 2010; Gaunt et al., 2013a; Patton et al., 2006). Secondly, concerning GLA supplementation, PGE_2 production appears to be increased and could play an immune-signalling role during infection, as PGE_2 is well known for its role during other viral infections (Sander et al., 2017). It is also possible that it is a combination of these two mechanisms that allow GLA supplementation to have a more significant effect than OA. Another possible explanation for the effect of OA on SA11 could be the central role of OA within fatty acid metabolism. OA can be used during β-oxidation (Houten and Wanders, 2010) of fatty acids to induce an energy-rich environment that the virus can use to fuel viral replication (Greseth and Traktman, 2014; Heaton and Randall, 2010).

In future work, the exact mechanism that SA11 uses OA to increase replication should be investigated. This can be done by inhibiting several enzymes that can metabolise OA, or by monitoring the production of ATP to determine if SA11 induces a hyper-induced energy state. Furthermore, the precise role of PGE_2 during SA11 replication needs to be elucidated as well as how SA11 increases the production of PGE_2. This can be done by inhibiting several enzymes within the biosynthetic pathway such as phospholipases, cyclooxygenases and prostaglandin E syntheses or by determining which transcripts are activated by the SA11-induced PGE_2. Furthermore, the possible interplay between PGE_2 and Ca^{2+} signalling (Koukoui et al., 2006; Rajagopal et al., 2014) should be studied to determine the role this interplay could have during RV infection. This could lead to the identification of possible pathways and/or proteins that can be targeted for the development of antiviral therapies to determine if they have inhibitory effects on rotavirus replication. Elucidating these

mechanisms, could possibly lead to an universal anti-viral treatment as several viruses, such as HIV and influenza, have been shown to use PGE_2 for their benefit during viral replication by increasing pathogenesis and replication (Coulombe et al., 2014; Liu et al., 2012; Olivier and Tremblay, 1998; Zambrano-Zaragoza et al., 2014).

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Animal Research Ethics

05-Dec-2017

Dear Mr Willem Sander

Student Project Number: UFS-AED2017/0069

Project Title: Fatty acids, eicosanoids and rotavirus infectivity

Department: Microbial Biochemical and Food Biotechnology (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on , the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.

A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.

Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.

Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

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Prof. Derek Litthauer Chair: Animal Research Ethics Committee

**agriculture,
forestry & fisheries**

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Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
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Reference: 12/11/1/4

Dr HG O'Neill

Department of Microbial, Biochemical and Food Biotechnology

University of the Free State

Tel: 051 401 2122

E-mail: HGONeill@ufs.ac.za; OneillHG@ufs.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Dr O'Neill

Your undated application form sent by email on 13 February 2018 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

2. The research project is approved as per the application form sent by email on 13 February 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

4. This Section 20 permit is only valid for sampling in the areas of the Free State province that have supplied a state veterinary letter of no restriction to the researcher and DAFF. No sampling may take place in any other area or province without written permission from the Director of Animal Health. Please apply in writing to HerryG@daff.gov.za;

5. This research project must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act no 19 of 82);

6. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project;

7. Only a registered waste disposal company may be used for the removal of all potentially infectious waste from the research project;

8. Records must be kept for five years for auditing purposes;

Title of research/study: Investigation into the effect of fatty acids on the yield of infectious rotavirus in MA104 cells

Researcher: Dr HG O'Neill

Institution: Laboratory of Molecular Virology in the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State

Permit Expiry date: 30 June 2018

Our ref Number: 12/11/1/4 (696KL)

Your ref: UFS-AED2017/0069

Kind regards,

(signature)

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -02- 1 6

**agriculture,
forestry & fisheries**

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Prof Hester Gertruida O'Neill
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University of the Free State
Bloemfontein
9301
OneillHG@ufs.ac.za
christiaan@deltamune.co.za

Dear Prof O'Neill,

RE: AMENDMENT ON PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your email of 2018-07-19, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other Act of the Republic of South Africa;

2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study.   Records must be kept for five years for audit purposes.   A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;

3. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under the Section 20

permit and this amendment letter. Please apply in writing to HerryG@daff.gov.za;

4. NSP2 and NSP4 antibodies, produced in rabbits, may be obtained from Deltamune and transported to the Molecular Virology Laboratory at the University of the Free State for immunohistochemistry;

5. The rabbit antibody samples obtained from Deltamune may be stored in the freezer at the Department of Microbial, Biochemical and Food Biotechnology at UFS, however any further use or distribution of the samples will require a new Section 20 permit;

6. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Investigation into the effect of fatty acids on the yield of infectious rotavirus in MA104 cells

Researcher (s): Prof Hester Gertruida O'Neill

Institution: Department of Microbial, Biochemical and Food Biotechnology, University of the Free State

Your Ref./ Project Number: UFS-AED2017/0069

Our ref Number: 12/11/1/4

Expiry date: 2019-02

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -10- 1 7

SUBJECT: SECTION 20 AMENDMENT FOR: INVESTIGATION INTO THE EFFECT OF FATTY ACIDS ON THE YIELD OF INFECTIOUS ROTAVIRUS IN MA104 CELLS LJvR

Tissue culture infection doses

1. **Introduction**

The procedure is performed to determine the infectious titer of any virus which can cause cytopathic effects (CPE) in tissue culture over a reasonable period of 5 to 20 days while cells in culture remain viable. This procedure is performed to quantify how much infectious virus is in a preparation. Not all virus types cause CPE in tissue culture, and the cell line and virus must be carefully matched in order to see a cytopathic effect. The TCID50 is determined in replicate cultures of serial dilutions of the virus sample. The titer of the virus stock is expressed as the TCID50 which can be calculated using a statistical Excel program and is more accurate than a negative end-point.

2. **Principle/Scope**

This SOP gives clear direction to all staff and students on how to preform $TCID_{50}$ assays.

3. **Safety**

• Foetal calf serum may contain infectious agents so appropriate PPE must be worn when working with FCS or media containing FCS. This includes lab gown, safety glasses and gloves. Heat inactivation of FCS limits exposure to infectious agents
• Cells in culture especially transformed cells should be assumed to potentially contain infectious agents. Appropriate PPE must be worn when working with cells including lab gown, safety glasses and gloves.
• Cell culture should be performed in a class II Biohazard hood to protect the product and the operator
• First aid procedures are as follows:
  ▪ Eye contact: flush with water and seek medical advise
  ▪ Skin contact: Wash off with water and mild soap/detergent, seek medical advise
  ▪ Ingestion: Seek medical advice
• If spillage of FBS, cell culture media or cells occurs spray with 80% ethanol and wipe up with paper towel or tissues. Dispose of by autoclaving
• Cell culture and maintenance requires the use of glass pipettes. Appropriate technique must be used to avoid injury from breaking of glass pipette. This includes the use of pipette boys and inserting the pipettes into the pipette boy by the correct method. Mouth pipetting is banned.

4. **Licenses and Permits**

5. **Training and Competency**

- Understanding of and instruction in the use of Class II Biohazard hood
- Understanding and instruction in aseptic technique
- Understanding and instruction in the use of glass pipettes
- Understanding and instruction in the calculations in $TCID_{50}$

- Training must be carried out by an approved operator and recorded in laboratory training manual. An approved operator is a member of the lab who is competent in the procedure.

6. Risk Assessments

7. Equipment and Maintenance/Handling and Storage/Labelling

EQUIPMENT:
Cell culture media and some reagents are kept in the TC fridge
Class II Biohazard hood and all other equipment required is located within the lab
The hemocytometer is stored within TC

MAINTENANCE:
N/A

HANDLING & STORAGE:
N/A

8. Operating Procedures

Equipment required:
Certified Biological Safety Cabinet
Micropipette and sterile tips
Inverted microscope
Tubes for dilution of virus
Hemocytometer with coverslip
CO_2 incubator set at 37 $^\circ$C
12 well plates
Culture flasks

Reagents required:
Infection DMEM
Complete DMEM
Trypan Blue Solution
PBS

8.1. Day before hand:
One day previous to infection, prepare 12-well dishes by seeding each well with 7 x 104 cells in 3 ml Complete DMEM.
8.2. Viral Dilutions:
On the day of infection, make dilutions of virus sample in infection DMEM
(NOTE: some viruses require extra reagent. E.g. rotavirus require trypsin)
Make a series of dilutions at 1:10 of the original virus sample.
Vortex virus sample and dilute as follows:

Dilution	Cell control	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Vol of MEM	1 mL	1 mL	1 mL	7.2 mL	7.2 mL	7.2 mL	7.2 mL	7.2 mL
Vol of virus	None	10 µL	10 µL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL

8.3. Addition of Viral dilutions

Label lid of 12-well dish by drawing grid lines to delineate quadruplicates and number each grid to correspond to the virus sample and label the rows of the plate for the dilution which will be plated.

10^{-6}		10^{-7}	
Control			

10^{-8}		10^{-9}	
Control			

Remove all of complete DMEM from each well by vacuum aspiration and wash with PBS.

Starting from the most dilute sample, add 1 ml of virus dilution to each of the wells for that dilution. NOTE: Be careful not to track the pipette tip over the wells, and continue infecting the plate with 0.1ml of virus dilution per well, wells per dilution, proceeding backwards through the dilutions.

Allow virus to adsorb to cells at 37 ºC for 2 hours or at a temperature indicated for the specific virus being tittered

After adsorption, remove virus inoculum with vacuum, beginning by sucking the most dilute wells and proceeding backwards to less dilute.

Add 0.5 ml Infection Medium to each well. Do not touch the wells with the pipette.

Place plates at 37 ºC, depending upon instructions and monitor CPE using the inverted microscope over a period of one to four weeks, depending on the cultural characteristics of the virus in question.

Record the number of positive and negative wells.

8.4. Calculation:

Calculate the proportionate distance (PD)

$$PD = \frac{(\% \text{ next to above } 50\%) - 50\%}{(\% \text{ next to above } 50\%) - (\% \text{next to below } 50\%)}$$

Calculate the 50% end point   this is the first dilution that is below 50%
Add the PD and Log lower dilution   PD + 50% end point
Calculate the TCID$_{50}$/ml   Divide by the ml of viral inoculum added
Convert to PFU/ml   pfu/ml = 0.7 * TCID$_{50}$

8.5. Example

Table 1 Calculation of virus titre in mice using the Reed and Muench method

Log$_{10}$ virus dilution	Mice		Cumulative total			Percent mortality
	Died	Survived	Died	Survived	Total	
-1	10	0	57	0	57	57/57 × 100 = 100
-2	10	0	47	0	47	47/47 × 100 = 100
-3	10	0	37	0	37	37/37 × 100 = 100
-4	10	0	27	0	27	27/27 × 100 = 100
-5	10	0	17	0	17	17/17 × 100 = 100
-6	6	4	7	4	11	7/11 × 100 = 63
-7	1	9	1	13	14	1/14 × 100 = 7

Difference of logarithms = (63-50)/(63-7) = 0.23; log$_{10}$ 50% end point dilution = -6 - (0.23 × 1) = -6.23; 50% end point dilution = $10^{-6.23}$; the titre of the virus = $10^{6.23}$ LD$_{50}$/mL.

Convert to PFU/ml = 0.7 x $10^{6.23}$

8.6. Cleaning

Unused sterile media is stored at 4˚C in laboratory fridge

Unused PBS stored is stored at 4˚C in laboratory fridge

Liquid waste to be poured into waste beaker and disinfected with 1% virkon and UV for 15 min and then discarded in biohazard box

All used culture flasks to be thrown in biohazard box

APPENDIX C: FIGURE PERMISSION

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