Sophorolipid-mediated Inhibition of Adenoma Development in Familial Adenomatous Polyposis Syndrome.

By

Breedge Callaghan MBMSc

School of Biomedical Sciences

Faculty of Life and Health Sciences

Ulster University

A thesis presented to Ulster University for the degree of Doctor of Philosophy

October 2017

I confirm that the word count of this thesis is less than 100,000

Caroline,

My sister and guardian angel,

I dedicate this thesis to your memory.

Contents page

Acknowledgements

"Alone we can do so little, together we can do so much"

This has been a journey containing many highs and lows but it is not without the support that I had, that I would be able to write this thesis today. I would like to firstly thank Prof. Christopher Mitchell who has been behind me since my undergraduate days and without his help, patience and kindness, I would not be the scientist that I am today.

To Prof Ibrahim Banat and Prof Roger Marchant, thank you for all the support over the past three years. I will never forget the excitement in your eyes, Ibrahim, when I presented new finding to you. The passion and enthusiasm for science you portray is admirable.

I would like to thank all the technical and academia staff in the CMB and especially Rob, Davey & Lisa in stores for always being there to help, offer advice and have the banter with. To all the friends I have made in the vascular biology group and beyond, especially Paul, Aine and Patricia. You guys have been the best office mates a girl could ask for, through the laughter and the tears, you were always there for me. To Laura, my pain buddy, – you have been my rock and I don't think I could have got through this without our friendship and numerous encouragement/counselling sessions – you are one in a million, there are not enough words to describe my gratitude!

To my family at home in the hills of Donegal- mum, dad, Stephen and granny B. No words can describe how thankful I am for everything you have done to bring me to this point in life. You always understood when I didn't get home as often as I should have and through everything, encouraged me to blossom to the person I am today. You are truly amazing people, both inside out.

To my extended family – Breige, Dermot and especially Órla, thank you for being there to listen, make me smile and reassure me that there was light at the end of the tunnel! I will be proud to bear the Conwell name.

I must sincerely thank my wonderful friends at St. John; Brian, Hannah, Fiona, Craig, Andrew and Amanda. You have kept me sane (even though I may have driven you insane) through this whole process. You all will always hold a special place in my heart.

Saving the most important to last, Michael, Sophia and Clio. You three are my everything. Michael, you have been by my side from the very first day of our first year of university. Who would of thought, through an undergrad, masters and PhD, we would follow in each other's footsteps and become husband and wife (to-be). Miss Sophia, you give my day's purpose and are the reason I push myself to finish this thesis.

Céad míle fáilte

Summary

Sophorolipids (SL) are amphiphilic biosurfactant molecules consisting of a disaccharide sophorose with one fatty acid at the C1 position and optional acetylation at the C6'and C6" positions. They exist in a closed ring lactonic (LSL) or open acidic (ASL) structure. Sophorolipids are produced in crude mixtures in economically viable amounts by the yeast *Starmerella bombicola* and used in a variety of consumer products. Varying levels of anti- proliferative and anti-cancer activity of crude sophorolipid mixtures are described in a number of tumour cell lines *in vitro*. However, significant inter-study variation exists in the composition of SL species as well as other biologically active compounds in these mixtures, which makes interpretation of *in vitro* and *in vivo* studies difficult. It is hard to truly assess the anti-cancer action of SL due to the lack of *in vivo* studies. To date, only one other study evaluates the ability of a crude SL preparation to reduced tumour growth in a cervical cancer xenograft model. This thesis aims to evaluate the ability of two SL congeners, produced by *Starmerella bombicola*, which have been highly purified and well characterised against the colorectal cancer cell growth *in vitro* and the ability to regulate the development of pre-cancerous polyps *in vivo*.

This thesis first evaluated and compared the biological activity of a 96% pure preparation of C18:1 LSL and a 94% pure C18:1 ASL preparation against CRC cells (HT29, HT115, HCT116, Caco2, LS180) and two normal cell lines CCD-841-CoN (colonic epithelial) and MRC5 (lung fibroblast) *in vitro* by assessing their ability to modulate cell viability via a MTT assay and the regulate the metastatic properties of these cells by assessing changes to migration and invasion in culture. The overall consensus of the data suggest that LSL induce cytotoxicity in CRC cells but also induces cytotoxity in the normal colonic CCD-841-CoN

cells at lower concentrations; results are detailed further in chapter three. In comparison, ASL reduced the viability of the CRC cell lines at low concentrations but proved non-toxic to the normal colonic cell line; results of these studies are detailed further in chapter four.

In the latter portions of chapter three and four respectively, the biological activity of both congers were assessed in *wt* c57 mice and $Apc^{min+/-}$ mice, a recognised animal model of Familial adenomatous polyposis (FAP) by first ensuring both LSL and ASL were well tolerated at doses of 0.5, 5 and 50mg/kg when administered orally. Next the ability of SL congeners to mediate adenomatous polyp growth was measured by counting the number of polyps present in the intestinal tract of $Apc^{min+/-}$ after 70 days of SL treatment compared to the vehicle-only treated control mice. In addition, parameters such as spleen weight and haematocrit levels were also measured as a method to evaluate the ability of SL to regulate the secondary side-effects associated with reducing the life-span of the $Apc^{min+/-}$. In summary, LSL resulted in exacerbated polyp growth in $Apc^{min+/-}$ with a further decrease in haematocrit in addition to splenomegaly. ASL had no effect on tumour number of size but increased haematocrit levels and reduced the spleen size of the treated $Apc^{min+/-}$ mice compared to vehicle control.

The final experimental chapter of this thesis explored the possible mechanisms of action of SL *in vitro*. The CRC cancer cell line HT29 was used to assess SL induced alterations to lipid raft expression, changes to tight junctions and SL congener specific changes to mitochondrial membrane potential and reactive oxygen species production *in vitro,* as described in chapter five.

In summary, contrary to current literature, purified ASL appears to have an advantage over the LSL as a potential therapeutic agent due to its ability to selectively reduce viability of

CRC cells without inducing toxicity at equivalent doses in normal colonic epithelial and lung fibroblasts form *in vitro*. Although ASL did not disrupt the tumour growth in the $Apc^{min+/-}$ mice, they improved the haematocrit levels and decreased spleen size which could result in an increase in life-span to these mice as they are known to succumb to the effects of intestinal bleeding in the 5^{th} – 6^{th} month of life. To date, this is the first study to investigate the effects of a highly purified LSL and ASL preparation across a wide range of CRC cells *in vitro* and the first study to investigate the anti-cancer of purified preparation *in vivo.*

List of abbreviations

Abbreviation	**Meaning**
AO/EB	Ethidium Bromide/Acridine Orange
AOM	Azoxymethane
APC	*Adenomatous Polyposis Coli*
$Apc^{min+/-}$	Adenomatous Polyposis Coli, Multiple Intestinal Neoplasm heterozygous transgenic mice
ASL	Acidic Sophorolipids
BfSL	Bolaform SL
BS	Biosurfactants
C16-18	Carbon 16-18
Ca^{2+}	Calcium ion
CIN	Chromosomal Instability
CMC	Critical Micelle Concentration
CRC	Colorectal Cancer
CS	Cowden Syndrome

CTxB	Cholera Toxin Subunit B
DMH	1,2-Dimethylhydrazine
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia Coli*
ECM	Extracellular Matrix
EE	Ethyl Ester
EED	Ethyl Ester Diacetate
EEM	Ethyl Ester Monoacetate
ENU	Ethylnitrosourea
FAP	Familial Adenomatous Polyposis
FOLFOX	Folinic Acid, Fluorouracil And Oxalipatin
GL	Glucolipids
GRAS	Generally Recognised As Safe
HIV	Human Immunodeficiency Virus
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HPLC	High Performance Liquid Chromatography
i.v	Intravenous

IgE	Immunoglobulin E
JPS	Juvenile Polyposis
LSL	Lactonic Sophorolipids
MAP	MUT-Associated Polyposis
ME	Methyl Ester
MEOR	Microbial Enhanced Oil Recovery
MMP	Mitochondrial Membrane Potiental
MMR	Mismatch Repair Proteins
MNNG	N-Methyl-N-Nitro-N-Nitrosoguanidine
MS	Mass Spectrometry
MSI	Microsatellite Instability
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NMU	N-Methyl-N-Nitrosourea
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PCBM	Peripheral Circulating Blood Monocytes

Pirc	Polyposis In Rat Colon
PJS	Peutz-Jeghers Syndrome
ROS	Reactive Oxygen Species
S. aureus	*Staphylococcus Aureus*
SL	Sophorolipids
SVZ	Subventrical Zone
ZO-1	Zonula Occludens-1

List of Figures

List of Tables

Note on Access to Contents

I DECLARE AS FOLLOWS:
That I am the author and owner of the copyright in the thesis and/or I have the authority of the authors and owners of the copyright in the thesis to make this agreement and grant Ulster University and/or the British Library a non-exclusive royalty free perpetual licence to make available the thesis in digitised format through the Institutional Repository and/or through the British Library via the EThOS system for the purposes of non-profit academic research, private study, criticism, review and news reporting, illustration for teaching, and/or other educational purposes in electronic or print form That if my thesis/dissertation does include any substantial subsidiary material owned by third-party copyright holders, I have sought and obtained permission to include it in any version of my thesis available in digital format via a stand-alone device or a communications network and that this permission encompasses the rights that I have granted to Ulster University and to the British Library.

That the digital version of the thesis that I am supplying is as approved by the examiners at the time of the award of my degree and as previously submitted to the Library in printed form. That I have exercised reasonable care to ensure that the thesis is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I understand that neither Ulster University nor the British Library have any obligation to take legal action on behalf of myself, or other rights holders, in the event of infringement of intellectual property rights, breach of contract or of any other right, in the thesis.

I wish to delay access to my thesis for two years for the following reason:

Thesis is due for publication, either as a series of articles or as monograph

Thesis contains commercially sensitive information, the release of which might prejudice the commercial interests of any person including the author, the University or an external company

IT IS A CONDITION OF USE OF THIS THESIS THAT ANYONE WHO CONSULTS IT MUST RECOGNISE THAT THE COPYRIGHT RESTS WITH THE AUTHOR AND THAT NO QUOTATION FROM THE THESIS AND NO INFORMATION DERIVED FROM IT MAY BE PUBLISHED UNLESSTHE SOURCE IS PROPERLY ACKNOWLEDGED

Chapter 1

General Introduction

Thesis overview

The aim of this thesis was to investigate the anti-cancer potential of two purified sophorolipid congeners against colorectal cancer growth *in vitro* and *in vivo.*

This literature review gives an insight into the origin, production and current uses of biosurfactants with a specific focus on sophorolipids, a glucolipid produced by the yeast *Starmerella bombicola,* are reviewed.

We explore and discuss the potential use of sophorolipids in biomedical applications focusing on the biology and interactions of sophorolipids as anti-cancer compounds. This introduction provides a comprehensive review on the development of human colorectal cancer, in addition to comparing and contrasting the use of human cancer cell lines *in vitro* and relevant animal models. In particular, we detail the use of the $Apc^{min+/-}$ mouse to determine whether purified preparations of sophorolipid congeners inhibit growth and progression in this pre-clinical model of FAP progression.

1.1 Surfactants

Surfactants, a term coined in the 1940's (Gerhard and Heinz, 1940), are chemical products which have surface-interface tension reducing activity (Young and Coons, 1945) and are predominantly derived from petrochemicals (Deleu and Paquot, 2004). Surfactants are further classified by charge: being anionic, cationic, non-ionic or zwitterionic (Kume et al., 2008). The majority of surfactants are non-ionic, followed closely in total numbers by anionic which have greater functional properties compared to the cationic and zwitterionic, which are restricted to comparatively smaller niche markets (Deleu and Paquot, 2004).

The functional properties of surfactants include their use as detergents, emulsifiers, foaming agents, lubricants, wetting agents, rheology modifiers and starch retrogradation inhibitors (agents which reduce the staling process of baked goods) (Jacobson and BeMiller, 1998). Surfactant products are produced on a world-wide scale (10 million tons per annum) (Zana and Xia, 2003), with approximately half used in laundry and house hold detergents [4.9 million tonnes] and the remainder employed in the chemical [0.1], food [0.3] (Garti, 2002), textile [0.8], environmental [0.5], agricultural [0.6], health care and cosmetic industries [2.0] (Holmberg et al., 2003).

However, surfactants are petroleum based, with a major disadvantage being their lack of biodegradability and biocompatibility, which adversely affects the environment as they accumulate in water sources and cause toxicity to marine based organisms. (Schröder, 1993). The non-biodegradability of surfactants can cause contamination of soils and water sources which can be taken up by cows resulting in surfactant bioaccumulation in the beef – which is then eaten by humans. Beef contaminated with the surfactant perfluoroalkyl has been reported by Lupton *et al* and serve as a potential severe health risk due to its link with hepatocellular hypertrophy and reproduction dysfunction (Lupton et al., 2014).

In order to reduce the dependency on petroleum based surfactants, various formulations based on raw-material surfactants produced from fats, oil and organic carbon sourced from fossils were developed (Scott and Jones, 2000).

The use of surfactants derived from raw materials greatly reduced greenhouse gas emission levels compared to petrochemical sources (Patel, 2003). However, a study carried out under the European Climate Change Programme (ECCP) recommended that surfactants produced by micro-organisms would further reduce greenhouse gas emissions and improve biocompatibility (Schroeder, 2009). Thus, the search for naturally produced surfactants, which are economically viable and can be separated via extraction, precipitation or distillation from micro-organisms, plants, invertebrates or animals has been an area of intense research.

1.2 Biosurfactants

Biosurfactants (BS) are a class of natural surfactants produced from renewable resources *via* biotechnological methods and were first discovered in the late 1960's. BS are produced by a variety of microorganisms as secondary metabolites, forming emulsions that reduce both interfacial and surface tension (Mulligan, 2005) when grown on water-miscible or oily substrates. They are either secreted into culture broth during production or may remain adhered to microbial surfaces. BS are classified as either glycolipids, lipopeptides, phospholipids, fatty acids or polymeric compounds (Table 1.1). The natural physiological role for BS secreted by micro-organisms in the environment is not yet fully understood. It is hypothesised that they facilitate the growth of microbes on water immiscible substrates, increase adhesion to insoluble substrates or aid in the interaction with metals in the environment. Other studies have also shown that they play an important role in quorum sensing in bacteria (Ron, 2001).

BS have clear advantages over chemical surfactants which include increased biodegradability, low toxicity and the ability to exert their effects at extreme temperatures and pH levels (Rodrigues and Teixeira, 2010); they prove versatile for a wide range of biomedical and industrial applications (Fracchia et al., 2012). Currently, a range of microbial biosurfactants are used in cleaning supplies (Bognolo, 1999), pesticides (Finnerty, 1994), textiles (Montoneri et al., 2009) and cosmetics (Kralova and Sjöblom, 2009) while petroleum derived surfactants are still used in food products (Nitschke and Costa, 2007) and over-the-counter creams (Levin and Miller, 2011). BS have also been shown to have biological activity for use as antibiotics (Nielsen et al., 2002), fungicides (Stanghellini and Miller, 1997), anti-viral/immunoregulators (Vollenbroich et al., 1997) and enzymatic inhibitors (Cameotra and Makkar, 2004).

Table 1.1: Types of major BS			
Name	Type	Species Producing	Potential Applications
Lipopeptide (Morikawa et al., 1993)	Surfactan	Bacteria- *Bacillus* sp.	Anti-microbial, anti-viral
Phospholipids (Todd et al., 2010)		Bacteria - *Thiobaccillus thioxidans*	Lowering surface tension, Anti-microbial
Fatty acids (Mulligan et al,.2001)	Corynomycolic acid	*Cornebacterium lepus*	Environmental
	Spiculisporic acid	*Penicillium* spiculisporum	Heavy Metal decontamination
Polymeric Compounds (Torchilin et al., 2001)	Emuslan/Alasan	*Acinetobacter* sp	Emisfying agent
	Liposan	*Candida lipolytica*	Emulsifier
Glycolipids (Banat et al., 2000	Sophorolipids	Starmenella Bombicola	Anti-microbial, anti-viral, anti-cancer
	Rhamolipids	*Pseduomonas* sp	Anti-microbial, environmental
	Mannosylerythriol	*Candida* sp.	Anti-microbial, environmental
	Trehalolipids	*Mycobacterium*	Anti-microbial, environmental

1.3 Sophorolipids

The sophorolipid (SL) class of biosurfactants were first described in the early 1960's by Gorin *et al,* as extracellular glycolipids synthesized from the non-pathogenic yeast – *Candida apicola* (Gorin et al., 1961*)*. In the same decade, SL synthesized from *Rhodotorula bogoriensis* and *Starmerella bombicola* were also discovered, proving that production is not limited to a singular species, but to a number of related micro-organisms (Spencer et al., 1970). SL have many attractive properties for large scale industrial production, such as low production costs, minimal toxicity and biodegradability (degraded 61% after 8hrs post-production).

Naturally found SL mixtures are made up of eight major components and composed of a hydrophobic fatty acid (C16- C18) tail and a hydrophilic carbohydrate head. The fatty acid tail can have one or more unsaturated bonds while the head is composed of a disaccharide sophorose with a β-1, 2 bond which is acetylated on the 6' and/or 6'' position. The structure of SLs is dependent on a terminal or sub-terminal hydroxylated fatty acid, which is linked β-glycosidically to the sophorose. The fatty acids' carboxylic end can be free, giving rise to the acidic structure (Fig 1a) (Cavalero and Cooper, 2003b) or can be esterified at the 4'' position giving rise to the lactonic ring structure (Fig. 1b).

Figure 1.1: The structure of SL

a

b

1.3.1 SL synthesis and production

SL synthesis relies on both a glucose and a fatty acid supply, which can be supplemented in the production medium. Biosynthesis of SL occurs as follows:

Fatty acids are converted to either a terminal or sub-terminal hydroxyl fatty acid. This occurs via membrane bound action of a NADP dependent mono-oxygenase enzyme – cytochrome P450. Next glucose is coupled glycosidically to the C1 position of the hydroxyl group of the fatty acid via glycosyltransferase I. This reaction requires a nucleotide-activated glucose as a glucosyl donor. A second glucose is then coupled to the C2 position of the first large glucose molecule via glycosyltransferase II. SL obtained at this point is classed as non-acetylated acidic molecules. A majority of the molecules formed continue on in the fermentation process and are further modified via esterification (lactonization) or acetylation of the carbohydrate head forming Lactonic SL. Most LSL are esterified at the 4'' position. A minute percentage is esterified at the 6' or 6'' position which is carried out by a further step involves the use of acetyl co-enzyme A (Coates *et al,* 2007) dependent acetyl transferase.

The process of biosynthesis of sophorolipids (described above) occurs during fermentation of *Candida bombicola* (Van Bogaert et al., 2007). An important aspect of fermentation are the culture conditions. The optimal temperature for the culture of SL producing strains is 28.8°C which produces the highest yields from the *Candida Bombicola* strain used in this thesis and allows the initiation of the exponential growth phase. A drop of pH from seven to two can often be seen here, however for high yield output at pH of 3.5 is best for our strain of SL producing *Candida Bombicola* (Roelants et al., 2016). This can be achieved with the addition of NaOH. It is important that there is a good supply of oxygen during the exponential phase of growth. An oxygen transfer rate of 50 – 80mM O_2/l h^{-1} is also a necessity for a high SL yield.

The production of SL is also highly dependent on the culture medium composition. The main components of culture medium needed for SL synthesis include glucose (first glycidic carbon source), yeast extract (nitrogen source) and urea (Casas and García-Ochoa, 1999). However, product yields can be greatly increased with the addition of a second carbon source of a lipophilic carbon source nature. This usually involves the addition of an oil or fatty acid such as vegetable oils (sunflower, safflower or soybean). Rapeseed oil was used as the secondary carbon source in this thesis as it produces a higher yield of SL (Daniel et al., 1998).

The final stage of SL production involves the extraction of SL from the culture broth which is a vital step in order to produce a highly pure preparation. This can be achieved by the addition of an organic solvent such as ethyl acetate. However, this allows residual lipidic carbon sources to also be extracted which can be problematic during purification. Therefore, hexane extraction is also employed (ethyl:hexane 1:1) (Baccile et al., 2013). Newer techniques applied to SL separation includes a simple centrifugation, as SL is heavier than water, excess medium can be simply decanted, which is beneficial for large volumes (Casas and García-Ochoa, 1999).

Over the years of SL production, *C. bombicola and Wickerhamiella domercqiae*; were classed as the preferred stain due to the high yield (400g/l). However further strain characterising tests revealed that the SL producing stain of *Wickerhamiella domercqiae* is actually *Starmerella bombicola* CGMCC 1576 (Chen et al., 2006, Li et al., 2016b, Li et al., 2016a). *C. bombicola* strains have now been listed as microorganisms that are technologically beneficial and generally recognised as safe (GRAS) (Bourdichon et al., 2012).

It has been proposed that the application of SL is directly related the structure of the mixture used (highly purified or high percentage of LSL or ASL) (de Oliveira et al.,

2015). One the attractive features of SL are their ability to be chemically modified. During the fermentation process, alongside LSL and ASL, four other SL derivatives are formed which differ by structure which include ethyl ester (EE), ethyl ester monoacetate (EEM), ethyl ester diacetate (EED), methyl ester (ME). A common modification occurs at the carboxylic end of the fatty acid forming alkyl esters (Azim et al., 2006, Sleiman et al., 2009). LSL have been shown to possess better biomedical properties such as anti-bacterial (De Rienzo et al., 2015), anti-cancer and spermicidal in comparison to ASL and SL derivatives but prove difficult to solubilise. ASL prove better in foaming agents such as cleaning agents and their solubility makes them more suitable in food and cosmetic applications (K Morya et al., 2013) as they can be dissolved in non-toxic solutions such as saline (Van Bogaert et al., 2007) while SL derivatives mixtures have also been shown to have strong anti-viral activity compared to both LSL and ASL (Borsanyiova et al., 2016).

It has been hypothesised that further investigation could lead to the production of SL congers to target specific diseases; i.e. LSL compounds as anti-bacterial agents, ASL for cleaning agents and derivatives for the use as anti-viral agents (Zhang et al., 2004).

1.3.2 SL applications

SL already have widespread uses in world today particularly in cleaning products and pesticides (de Oliveira et al., 2015). They are employed in the formulation of beauty and personal care products such as acne pads, anti-dandruff shampoo, lipstick and toothpaste (Shete et al., 2006). The use of SL are favoured over petroleum based surfactants due to their low toxicity and their potential biomedical enhancing properties including their ability to stimulate human cell growth such as fibroblasts, induce collagen production and activate macrophages for wound healing (Maingault, 1999). The main applications of SL in the commercial sector and for potential biomedical use are described below.

Agricultural, environmental and oil recovery uses of SL

SL mixtures derived from *Candida bombicola* with a high percentage (>50%) of ASL (de Oliveira et al., 2015) have biocidal properties against phytopathogens such as *Phytophthora sp* when used as an herbicidal adjuvant with lemongrass oil (LGO), an organic herbicide, due to their ability to adhere to the plant surface thus resulting in an increased penetration (Giessler-Blank et al., 2010). SL demonstrate anti-fungal potency against soil borne pathogens such as *Pythium sp.* which can cause root disease infecting seeds, leaves and stems via the formation of zoospores (Yoo et al., 2005). SL has the ability to inhibit mycelial growth and motility and can result in the lysis of the zoospores.

Although the process of oil production has been well-established over the years, there is still 67% of residual oil left behind in reservoirs after mining. To increase oil recovery, microbial enhanced oil recovery (MEOR) is applied to increase the overall quantity of oil harvested (Nerurkar et al., 2012). SL is attractive for the use in MEOR due to their ability to reduce interfacial tension resulting in improved drainage, reduction of oil viscosity and clearing oil flooded capillaries, increasing the quantity of oil harvested. SL can also aid in the cleaning up of oil spill related accidents due to their ability to bind and degrade

hydrocarbons such as 2-methylnapthalene and hexadecane in less than 7 days (Elshafie et al., 2015). SL has been shown to be one of the best non-ionic BS for soil washing due to its ability to degrade aliphatic and aromatic hydrocarbons by 85-97% under laboratory conditions (Kang et al., 2010).

Cleaning products

SL can be used in the production of detergents and stabilizers due to their ability to lower surface tension of liquids from 72 mN/m to 35mN/m (Ma et al., 2012). SL demonstrate the ability to form micelles, a key feature of surfactants, at low concentrations (e.g. 40-100mg/L, defined as the critical micelle concentration CMC), have a hydrophilic balance of 10-13 and foam forming capabilities. These characteristics render SL as a good wetting agent thus making them ideal for use as dishwasher agents and for cleaning hard surfaces (Develter and Lauryssen, 2010).

Food

Surfactants have been used in the food industry for many years in products such as salad creams and deserts as they can contribute to creaminess, texture and palatability. The use of natural BS such as SL is more appealing to the food industry as they can improve the quality of wheat flour by promoting volume, appearance and shelf-life of baked goods (Nitschke and Costa, 2007). The anti-microbial and germicidal properties of SL are also an attractive feature to the food industry. Formulations which include as little as 1% of SL have been shown to kill organisms such as *E. coli*, *Samonella typhi* and *Shigella dysenteriae (*de Oliveira *et al.*, 2015*)* which are known to play a role in food spoilage and food borne disease.

Anti-microbial

The antimicrobial properties of SL are not just limited to bacteria. They also demonstrate anti-mycoplasma and anti-algal properties (Van Bogaert et al., 2007). The extent of anti-microbial activity is dependent of the chemical structure of SL and structure of the target bacterium, for example, LSL show a greater extent of inhibition against gram positive bacteria such as *Bacillus subtilis, Staphylococcus epidermidis, Streptococcus faecium, and Propionibacterium acnes* all of which are associated with skin infections (Lang et al., 1989). Potency of LSL against these bacterial strains opens up the possibility of their use in skin creams, cosmetics and hygienic products (K Morya et al., 2013). It is believed that SL exert their anti-microbial effect *via* the destabilization of the bacterial membrane resulting in a change in permeability, thus allowing membrane rupture and expulsion of cellular contents (Azim et al., 2006).

The advantages of SL as a primary antimicrobial agent in comparison to current antibiotics used to treat clinical relevant bacteria are unclear. A number of studies has been carried out to test the antibacterial effect of SL. These tests are usually carried out in bacteria which classified as GRAS. An alternative solution has been suggested to combat the reduced potency of SL. Studies have shown the synergistic properties of SL when used as an antibiotic adjuvant enhancing the activity of antibiotics such as tetracycline and cefaclor against *S. aureus* and E.*Coli* (Joshi-Navare and Prabhune, 2013).

Anti-viral and spermicide

SL have shown great promise as spermicidal and anti-HIV agents (Gross et al., 2004) *in vitro*. These biological effects are highly dependent on their structure with short carbon

chain SL have a higher viral potency (C11-13), while long chain SL structures (C15-18) show anti-spermicidal activity LSL demonstrated superior spermicidal, cytotoxic and inflammatory activity however the addition of an acetyl groups lower the hydrophilicity of SL thus improving antiviral and cytokine stimulating effects. Unfortunately, SL mixtures also exhibit toxicity against the VK-2/E6E7, an epithelial vaginal cell line, at doses of 23µg/ml or greater when tested *in vitro* (Shah et al., 2005) which could prove problematic for possible clinical translation.

Immuno-modulatory

The use of SL as immune-modulatory agents has been widely researched in septic shock model's due to their anti-microbial, anti-viral and anti-inflammatory properties (Napolitano, 2006). The use of animal models of sepsis have become popular to test immuno-modulatory agents (Bluth et al., 2006). Gram negative bacterial-induced sepsis is exacerbated by the induction of a cytokine cascade which in-turn activates the coagulation and apoptosis cascade resulting in organ damage and intravascular coagulation (Cohen, 2002). The administration of SL mixtures in a rat model of sepsis decreased macrophage numbers, nitric oxide production and pro-inflammatory cytokines which is thought to contribute to the overall reduction in mortality rates (Bluth et al., 2006). Similarly, the administration of SL mixtures at concentrations ranging from 0.1-100µg/ml results in an immune-modulatory effect by reducing IgE production, both in U266 myeloma cells *in vitro* as well as in a mouse model of asthma *in vivo* (Hagler *et al.*, 2007).

Drug delivery systems

The ability of SL to self-assemble into different congeners as well as the extent of their amphiphilicity, (both of which are dependent on production methods, temperature and pH levels) makes them an ideal target for their use as a drug delivery system (Faivre and Rosilio, 2010). ASL can be used as a vehicle for mogroside V, a drug with promising anti-atherosclerotic, anti-cancer and anti-diabetic properties, enhancing penetration of the drug through the skin thus increasing its efficacy (Imura et al., 2013). SL have also been shown to enhance drug penetration. When coupled with Lactoferrin, a compound used to stimulate wound re-epithelialization when tested in human dermal fibroblasts (HDFn) *in vitro*, SL has been shown to increase the expression of tropoelastan resulting in increased cell proliferation and collagen synthesis in an *in vitro* model of transdermal absorption (Ishii et al., 2012). This also shows that SL is non-toxic to normal cells.

Anti-cancer

SL, isolated from *Candida bombicola*, have been reported to possess anti-cancer properties (Chen et al., 2006b), with crude LSL enriched preparations showing potent cytotoxicity against human liver (HT7402), lung (A549), breast (MDA-MB-231), cervical (HeLa & CaSki) and leukemic (HL60 & K562) cancer cell lines (Chen et al., 2006b). Crude preparations of SL with undisclosed LSL/ASL ratio percentages have also been reported to show dose-dependent, anti-proliferative and pro- apoptotic activity against pancreatic cancer (H7402, HPAC) and esophageal cancer (KYSE450, KYSE109) cell lines (Shao et al., 2012).

A number of hypotheses have been proposed to explain the mechanisms by which SL exert their anti-cancer potency; these hypotheses differ depending on which cell line is used. SL (defined by the authors as a mixture of LSL and ASL), induced differentiation of both leukaemia and glioma cell lines (Joshi-Navare et al., 2011) while Fu et al., 2008 demonstrated the induction of necrosis measured by LDH release in pancreatic cancer

HPAC cells *in vitro*. The cytotoxic effects of SL mixtures (with an undisclosed proportion of ASL/LSL congeners) have been observed in the liver cancer line H7402. This cytotoxic effect is mediated by blocking the cell cycle at G1 and S phase, increasing intracellular Ca^{2+} resulting in the induction of the caspase cascade and induction of apoptosis (Chen et al., 2006a).

Conclusions on the specificity of such diverse preparations of SL in transformed cells is complicated by the inappropriate use of controls – a number of studies have excluded any normal cell controls in their studies (Shao et al., 2012, Ribeiro et al., 2015, Li et al., 2016a) while other studies lack the use of appropriate primary or non-transformed cells which correspond with the tissue of origin for the cancer type used. Instead they opt for non-adherent cell lines such as peripheral blood mononuclear cells (PBMCs) in comparasion to pancreatic cancer cell lines (Fu et al., 2008) or Chang and co-workers, who used a HeLa contaminated cell line (Gao et al., 2011).

To my knowledge, only one published study investigated the anti-cancer potential of SL, where a cervical cancer (HeLa) xenograft model was used and mice were treated with a crude LSL mixture at concentrations of 5, 50 and 500mg/kg bodyweight. In this study, a crude SL mixture dose dependently decreased tumour weights and sizes after 12 days of intragastrical administration. Samples of tumours from mice treated with a dose of 50mg/kg LSL were shown to have a higher level of apoptosis compared to vehicle-only treated controls. However, the exact mechanisms of action in this aforementioned study are unclear (Li et al., 2016).

Limitations of SL description in the literature and study design issues

A wide range of bioactivities for SL have been documented, however the purity and composition of SL used in bioassays is highly variable, with most studies not disclosing the molecular species or their relative abundances within the mixtures (Fu et al., 2008,

Hardin et al., 2007, Chen et al., 2006b). Although LSL normally make up the highest proportion of crude preparations of SL (characteristically 70 – 85% (Manzke, 1999)), with varying amounts of ASL and other derivatives in the remaining mixture, this can change due to slight alterations in pH (Baccile et al., 2012) or temperature in the production process (Daverey and Pakshirajan, 2009, Amaral et al., 2010).

A major problem with the majority of SL related cancer studies available in the literature is the difficulty in comparing and contrasting the data due to the use of poorly characterised crude preparations. This also poses an issue with reproducibility of studies due to the difficulty of reproducing batches of similar composition which will also causes problems for the clinical translation studies (Harvey, 2008).

Another major issue of SL related cancer studies that populate the literature is the lack of follow through to *in vivo* model testing. With exception of our published study (Callaghan et al., 2016), only one other study has investigated the anti-cancer effects of SL *in vivo*. describing a xenograft model of cervical cancer and intragastrical injection of a crude SL mixture (Li et al., 2016a).

We proposed that due to the biosafety of SL, they could be administered orally. Therefore, we employed the FAP - $Apc^{min+/-}$ mouse model, which allowed the oral administration of a purified SL, giving direct access of the ingested SL to the growing of polyposis tumours along the intestinal tract.

1.4 Colorectal cancer

In the western hemisphere, Colorectal cancer (CRC) in both men and women is the fourth most common cancer diagnosed each year (~298,000 cases) (Center et al., 2009) and is the second most common cancer-related cause of death in the UK (Torre et al., 2016). CRC onset can be attributed to both genetic and environmental factors. The most common developmental contributions include familial inheritance of CRC disorders such as familial adenomatous polyposis (FAP) and epigenetic alterations (Kondo and Issa, 2004) while environmental factors such age (Nakagawa et al., 2001), poor diet (Vargas and Thompson, 2012), smoking/alcohol intake (Giovannucci et al., 1995) and lack of exercise (Samad et al., 2005) play a pivotal role in the development of CRC.

CRC's are defined as being highly heterogeneous at both the histopathological and molecular levels (Stigliano et al., 2014) and are classified as adenocarcinomas, squamous cell carcinomas, carcinoid, sarcomas or lymphomas. Clinical and histopathological analysis suggests that 90% of diagnosed CRC cases are adenocarcinomas arising from adenomatous polyps, originating in the intestinal lining, and are firstly benign before developing into carcinomas over a 60-80-year time period (Justin et al., 2015) (Neugut et al., 1998).

Theories designed to explain CRC pathogenesis include Foulds' hypotheses, stating that cancer is a multistep process developing over a long period of time (Foulds, 1965). Chronologically, this was followed by Nowells' Clonal Evolution Model, which hypothesized that the development of tumours is characterized by a number of repeated mutations causing a multi-clonal population which have the ability to invade surrounding tissue and subsequent metastatic disease (Nowell, 1976). This was followed with Volgelstein's theory detailing the adenoma-carcinoma sequence, giving the first real insight into the molecular pathways governing the pathogenesis of CRC (Fearon and Vogelstein, 1990). In this aforementioned model, specific genetic and epigenetic changes

were directly correlated to the initiation of neoplastic transformation of normal colonic epithelial to form benign adenomas before the development of a more malignant state (Fig 1.2). This model posits that mutations of four key genes (APC, KRAS, DCC, p53) can influence histopathological changes associated with clinical progression of CRC. The Loeb model closely followed in 1991, postulating that tumour progression is initiated by genetic instability resulting in the formation of a large number of random mutations and production of clonal clusters with a mutant phenotype (Loeb, 1991).

Fig 1.2: CRC advances from normal colonic epithelium, and via genetic alterations, adenomatous polyps are formed. These polyps have the potential to form adenocarcinomas and malignant metastatic tumours.

Vogelstein's group determined that CRC tumour development was initiated by destabilisation of the genome resulting in the inactivation of the tumour suppressor

Adenomatous polyposis coli (APC) gene – the first notable event in the CRC cascade which occurs in 60-80% of colorectal tumours (Powell et al., 1992).

The APC gene is found on chromosome 5q21 and plays an important role in cell adhesion, transcription as well as signal transduction and is a key player in the development of both sporadic and inherited CRC (CA44688 and CA41183, 1991, Fodde, 2002). The germline or somatic APC mutations present in 90% CRC tumours are found between codons 1286-1513 and result in early truncation of the protein (Cetta et al., 2007). In order to observe neoplastic transformations, both APC alleles must be inactivated. The APC protein plays an important role in the WNT signalling pathway which regulates β-catenin turnover in the cytoplasm. Any mutation, such as truncation of the APC protein, stimulates the over-production of β -catenin which can result in neoplastic growth (Näthke, 2004).

Although APC gene mutation initialises the start of the adenoma sequence, progression to carcinoma can only occur with the mutation of other genes. K-Ras mutations are found in 40% of CRC cases (Lievre et al., 2006). This oncogene plays an important role in cell proliferation. When combined with APC mutations, CRC progression only relies on one allele of the K-Ras gene to be mutated for the development of more advanced polyps with carcinoma potential (Fig 1.2).

The underlying origin of genetic mutations in CRC are believed to be caused by genomic instability (chromosomal instability (CIN)) which occurs in 85% of CRCs resulting in a loss or gain of chromosomal DNA or microsatellite instability (MSI) generating defective mismatch repair proteins (MMR) (Worthley and Leggett, 2010).

1.4.1 Types of colorectal cancer

CRC cases are categorised into sporadic (73%) or familial /hereditary (27%). A majority of sporadic cases occur with no underlying genetic origins yet they still share major similar genetic alterations as the inherited CRC forms.

Sporadic

Sporadic CRC account for 75% of all CRC cases and they usually occur in adults with a median age between 70-75 years (Hawk et al., 2015). In a majority of cases, CRC occurs without previous family history or a genetic predisposition. Sporadic CRC tumours predominantly develop at distal sites of the colon such as the recto-sigmoid region (Connell et al., 1994). In comparison to other cancers, CRC is relatively easily diagnosed and treated upon early diagnosis (Stewart and Wild, 2016). Similar to the inherited forms (see below), CRC are formed from benign precursor lesions known as polyps (Figure 1.2). As postulated by Vogelstein, these polyps develop into cancerous lesions under the influence of mutational changes brought about by age, diet, environmental or genetic factors.

Familial /hereditary colorectal cancer

Familial or inherited CRCs are caused by specific genetic alterations that result in patients being more susceptible to the development of adenomatous polyps. The most common familial conditions include Hereditary Non-Polyposis Colorectal Cancer (HNPCC) (5% of cases), MUT-associated polyposis (MAP) (1% of cases), Hamartomatous conditions and Familial Adenomatous Polyposis (FAP) (1% of cases)

Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

HNPCC, also known as Lynch syndrome, was first described in 1985 by Henry T. Lynch (Lynch and Lynch, 1985) and is an autosomal dominant disorder with incomplete

penetrance (Steinke et al., 2013). HNPCC is caused by mutations in the mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2 (Dunlop et al., 1997). HNPCC patients usually inherit one defective copy of the gene from their parents; however, it is not until the second copy of the gene is mutated that the syndrome occurs (Knudsons two-hit hypothesis (Knudson, 1996)). Mutations in both MMR genes such as MSH1 (Fishel et al., 1993) results in the malignant progression of adenomas in the colon. HNPCC accounts for 5% of all CRC cases and patients have an 80% risk of a CRC diagnosis in their lifetime (Stigliano et al., 2014). Patients with HNPCC can develop one or more adenoma tumours along the colorectal tract. These cancerous tumours are distinguishable from a sporadic tumour by simple molecular testing (investigating MMR and MSI gene alterations) (Herman et al., 1998). HNPCC patients present with CRC at around 45 years of age and have a high probability (approximately 30%) of developing another tumour within 10 years (Vasen et al., 2007). The histopathological presentation of HNPCC cancers are normally recognised as presenting as mucinous tumours which are poorly differentiated and favour growth on the right anatomical side of the colon (Steinke et al., 2013).

MUTYH-associated polyposis (MAP)

MAP, first reported in 2002, is an autosomal recessive disorder caused by biallelic mutations in the MUTYH gene and occurs in 1% of CRC cases (Sampson and Jones, 2009). The MUTYH gene plays an important role in base excision repair system which protects genomic DNA from oxidative related damage (Barzilai and Yamamoto, 2004). MAP patients do not have any germline APC mutations; instead they harbour germline mutations in the MUTYH gene which results in the growth of 10-100 polyps, which are primarily located in the proximal colon. These polyps begin developing in affected individuals at around 50 years old and have a 43-100% chance of progressing to

adenocarcinomas (Fleming et al., 2012). MAP patients usually present with clinical symptoms that are similar to other familial disorders such as FAP and HNPCC.

Hamartomatous conditions

Hamartomatous polyposis syndromes account for 1% of CRC cases. Hamartomatous polyps are focal malformations which are similar to a neoplasm but occur due to a development abnormality of the attached organ. The term is used to cover a unique group of inherited CFC forms which develop hamartomatous polyp's rather than the conventional epithelial polyps. This class of tumours includes Juvenile Polyposis (JPS), Peutz-Jeghers syndrome (PJS), Cowden syndrome (CS). Less common hamartomatous conditions include Bannayan-Riley-Ruvalcba syndrome and Cronkhite-Canda syndrome (Zbuk and Eng, 2007). Like the other CRC familial disorders, hamartomatous conditions can arise due to underlying genetic alterations involving SMAD-4 (JPS), LKB_1 (PJS) and PTEN (Cowden syndrome) (Wirtzfeld et al., 2001).

Familial Adenomatous Polyposis (FAP)

FAP is the second most common inherited colorectal cancer syndrome and occurs in approximately 1/10,000 people (Ruys et al., 2010). FAP is an autosomal dominant disorder with typical clinical presentation occurring in adolescence that includes the development of hundreds to thousands of large adenomatous polyps, coupled with severe colonic and rectal bleeding as well as severe anaemia. Although initially asymptomatic, these polyps invariably develop into colorectal cancer by the age of 50 (Bisgaard et al., 1994) without medical intervention. However, due to the new age of medical surveillance in patients at risk of polyp development, only 1% of cases progress to colorectal cancer (Bonnington and Rutter, 2016). Although polyps are predominantly found in the colon of FAP patients, primary tumours can also be found in the small intestine with secondary tumours located in the liver, brain and thyroid (McConnell, 1976).

The majority of patients diagnosed with FAP have a family history of colorectal cancer however 20-30% of cases are diagnosed with no previous clinical or genetic familial association (Giardiello et al., 2014). Medical surveillance occurs in patients with a family history of FAP from as young as ten years old and includes colonoscopy, polyp removal and prophylactic colectomy to dramatically reduce development and mortality rates associated with colorectal cancer secondary to FAP (Syngal, 2015). Although medical intervention in FAP cases is required to prevent disease progression, the specialist consultations and clinical/surgical interventions that are required results in a significant financial burden to the NHS. This emphasises the need for a cheap and easily produced pharmacotherapeutic agent to prevent or reduce the number of polyps which form in FAP patients.

Investigations of cancers with familial associations, have given insight into the mechanisms governing the development of sporadic colorectal cancer allowing the development of biomarkers and therapeutic targets. The most widely used methods for developing and testing therapeutic agents and their interaction with CRC progression involves the use of *in vitro* cultures (Cayrefourcq et al., 2015), human biopsies and tumour xeno-transplants (Tentler et al., 2012). Although tumour xeno-transplants are invaluable methods for scientific discovery, the major criticisms of their use are their inability to fully mimic progression of CRC as they do not grow within the natural hosts organ environment and often do not metastasise as they are most often implanted subcutaneously (McIntyre et al., 2015).

1.4.2 Experimental models of CRC

In order to determine a consistent response to potential therapeutics, is important to use a large panel of tumour-derived human cell lines from the same location (e.g. lung, pancreas or colon) which comprise different stages of the tumour development. However, the use of cell lines alone does not mimic the natural progression and development of colorectal cancer tumours as they lack key features of the natural micro-environment including cell-matrix interactions, stromal involvement or chemical/genetic influences. This emphasises the need for *in vitro* studies to be corroborated with an appropriate animal model (Richmond and Su, 2008). The ability to choose an appropriate animal model to characterise the effect of a chemotherapeutic, one must fully understand the pathogenesis of the disease.

1.5 Animal models of CRC Syndromes

Over the past 80 years, many models of colorectal cancer have been developed using different methods which include diet/chemical/mutagen induced (Liu et al., 2012, Chong, 2014), sporadic (Meyskens et al., 2008), xenograft (subcutaneously and orthotopic) (Hoffman, 2015). The main aim of an *in vivo* animal model of CRC is to mimic the molecular aetiology, pathology and clinical presentation in humans as close as possible. However due to the diverse causes of CRC, it is impossible for a sole model to encapsulate every aspect of the disease. Therefore, it is important, for translational purposes, if the following characteristics are present: tumour development limited to the large intestine, histologically and molecular progression similar to the human disease and should have similar cellular interactions (ie not immune compromised).

Several animal models which falls within a majority of the criteria above and are widely used can be grouped in three categories: spontaneous intestinal cancer models, exogenous promotors of CRC or genetic manipulation and mutagen-induced germline mutations.

Models of spontaneously occurring CRC

Intestinal cancer models in canines and sheep recapitulate human pathophysiology of CRC as tumours also occur in the large intestine, with pedunculated adenomas appearing in the distal colon/rectum and sessile tumours occurring in the proximal colon (Simpson, 1972). However, the use of both dogs and sheep has severe limitations including the high cost, specialised animal handling needs and the low prevalence of disease presentation (Church et al., 1987). Rodent models of spontaneous CRC development are preferred due to their low cost and ease of reproducibility. A spontaneous form of CRC has been reported in a very small number of C57BL mice, though this is extremely rare. Here a small number of adenomas formed along the small intestine spontaneously in a mouse without the use of genetic alteration or xenografting. However due to the rare occurrence

of spontaneous CRC development in mice it is not a viable option to meet the demands of medical research (Rowlatt et al., 1969).

Exogenous promotion of CRC

Mice or rats which are fed a diet that is high in fat (>20%), low in calcium (<0.05%) and low in Vitamin D (<100 lU/kg) that mimics a "western world diet" and leads to pre-cancerous changes associated with CRC. After 12 weeks on a Western diet, mice present with hyper-plastic crypts and two years' post-treatment show histological evidence of crypt dysplasia, polypoid lesions and an atypical nuclear epithelial phenotype (Newmark et al., 1990) which are also seen in CRC patients (Roncucci et al., 1991, Kiesslich et al., 2004, Sánchez-Tilló et al., 2011).Unfortunately, rodents are also subjected to a diet defect in calcium ($1/10^{th}$ of the dietary requirement) therefore concluding they may also undergo other physiological changes not relevant to CRC.

Chemical: The administration of carcinogenic agents to rodents have proved a common method of cancer induction. Both 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) (Wood et al.) are two commonly used agents, which when injected intraperitoneally or subcutaneously over six weeks induce CRC in rats. Rodents treated with these alkylating agents develop tumours in the colon after one year (DuBois et al., 1996). A majority of the tumours collected have β-catenin, K-Ras mutations and microsatellite instability (De Robertis et al., 2011) which is also commonly seen in both sporadic and HNPCC patients (Bissahoyo et al., 2005, van Zeeland, 1996).

A second combination of carcinogens known as N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (NMU) that can induce sporadic CRC in rodents when injected into the rectum for 5 weeks (Narisawa et al., 1974) . NMU-induced CRC is a relatively fast acting model (15 weeks vs 52 weeks AOM) which induces tumours that are genetically (K-ras mutations) and histopathological (aberrant crypt foci) similar

to humans (Bara et al., 2003). However, it must be injected intrarectally as oral administration (Maekawa et al., 1988) can cause neoplasms to also develop in the prostate (Boileau et al., 2003), kidney (Sharma et al., 1994), lung (Schoental and Magee, 1962), breast (Chander et al., 1994) and thymus (Newcomb et al., 1988).

Genetically modified mice

The use of genetically modified mice is useful to study the underlying genetic alterations which must occur for CRC development. These models can be designed to specifically target and control the type and location of the tumour growth by controlling the regulation of specific genes using transgenic mice (Madison et al., 2002, Saam and Gordon, 1999). This method of CRC induction has been previously used to study the initial stages of adenoma development by employing the Cre-*loxP* system. This introduces two *loxP* sites into the APC gene at introns 13 and 14 and introduced into embryonic stem cells. This resulted in the formation of a pair of homologous recombinant embryonic stem cells clones which allowed the development of a transgenic mice model via blastocyst injection. These chimeras can be breed to form a transgenic mouse colony which develop adenomas in the small intestine (Shibata et al., 1997). These models prove beneficial in studying the link between genes and tumour development however, the development of the model is time consuming and requires experienced personnel.

Mutagen-induced murine models

The use of mutagen-induced models of CRC have become the favourite and most used model for pre-clinical research due to their molecular (alterations in APC gene) and pathogenic (multiple neoplasm development) similarity to human CRC cases. The *i.p.* injection of ethylnitrosourea (ENU) in C57/BL mice resulted in the development of the $Apc^{min+/}$ mouse model by inducing a germline mutation in the APC gene (Moser et al.,

1995). These mice develop mutations in the APC gene resulting in the growth of numerous polyps along the intestinal tract (McCart et al., 2008).

Injection of ENU into rats also resulted in the development of the F344- Pirc (polyposis in rat colon) model, with 100% of affected animals developing intestinal polyps and progression to adenocarcinoma. Unlike the mouse model, Pirc rats develop equal numbers of polyps in the colon and small intestine (Amos-Landgraf et al., 2007), although it is not readily available for purchase by cancer researchers.

Although there are a wide range of animal models available to study the dietary, genetic and environmental changes that lead to CRC, the mutation-induced $Apc^{min+/-}$ model have proved to be most useful as it has a relativity fast experimental turnover (<150 days), reproducible, cheap and mimic neoplastic development seen in familial CRC conditions – FAP and HNPCC. They both have their benefits however the $Apc^{min+/-}$ mice are readily available to buy and does not require the use of hazard carcinogenic compounds results in a shorter experimental time frame, making it ideal to test potential chemotherapeutic compounds (Johnson and Fleet, 2013).

1.5.1 $Apc^{min+/-}$ mouse model of FAP

Although there are over 300 unique mutations in the human Apc gene that cause FAP, they all result in truncation of the APC protein and this mutation is an early event (pre-adenoma stage) in the pathogenesis of both familial and sporadic cancers (De la Chapelle, 2004). Adenomatous polyps are primary precursor lesions for the development of colorectal cancer in humans, thus making them attractive targets for chemotherapeutic agents.

The $Apc^{min+/-}$ mouse model of FAP was first genetically engineered in the 1990's by Moser (Moser et al., 1995) and provides an excellent *in vivo* system of the human disease and its development. It has become one of the most widely used models to investigate the effects of genetics (cMyc and ERK signaling) (Ignatenko et al., 2006) , diet (high fat foods) (Baltgalvis et al., 2009) and therapeutic drugs (Piroxicam and NSAIDs)(Jacoby et al., 1996) on tumorigenesis in the gastrointestinal tract. Moser et al, first produced the $Apc^{min+/-}$ mouse model by inducing the germline mutation with the use of ENU. This is a compound which can induce forward germline mutation in each locus by an occurrence of 1:700 resulting in gain or loss of function mutations. They detected this mutation in C57BL/6J (B6) male mice injected with ENU and mated them with AKR/J female mice and the female off-spring (F1) were subsequently mated again with a wild-type C57Bl/6J B6 male (F2 generation). A number of the off-spring (~25%) from these mice developed adult-onset anaemia with haematocrit levels <45%. It was noted that these mice did not have a long life span and at the point of death, haematocrit levels were 10-20%, with lethality being a consequence of severe anaemia. Later studies have shown that anaemia is evident from as early as 60 days (12 weeks) of age (Gould et al., 2003) and may be used as an early indicator of disease progression. It was also noted, upon autopsy, that $Apc^{min+/-}$ mice develop splenomegaly (Wood et al., 2008) and lymph node (Puppa et al.,

2011) enlargement near the end of their lifespan. The passing of bloody stools from 140 days+ was also observed in $Apc^{min+/-}$ mice and at the peak of their life-span, protruding tumours were viable form their anus. On post mortem, numerous tumours were found along the small intestine – ranging between 1-8mm in size (Moser et al., 1995).

Apc model alternatives

Following the development of the $Apc^{min+/-}$ mouse model, researchers produced genetically modified versions of the APC model replicating the different genotype-phenotype changes seen in human CRC such as polyps that were specific to the colon only compared to the $Apc^{min+/-}$ mice which developed them in the small intestine only (Corpet and Pierre, 2003). These genetically modified models are created to include mutations in other regions of the APC gene. For example, coupling a truncation mutation at the 850 amino acid region of the APC gene ($APC^{850)}$ with Msh2 mutations results in mice bearing 160+ polyps in the small intestine and 5+ polyps in the colon compared to the 40+ in the small intestine seen in the $Apc^{min+/-}$ mouse model (Karim and Huso, 2013). Use of the different types of Apc models allow researchers to manipulate polyp onset (Apc^{580} develop polyps within 4 weeks) and polyp origin (Apc^{1638N} polyps found primarily in the colon) (Fodde et al., 1994). Although there are different variations of the $Apc^{min+/-}$ mouse model, as mentioned above, the $Apc^{min+/-}$ is still the most widely used as it is commercially available to purchase and well characterised. The location of the tumours in $Apc^{min+/-}$ mice along the intestinal tract opens it up to orally administered drugs which, in theory, allows the drug direct access to the developing tumour on the epithelial surface.

SL haven been shown to be safe, non-toxic and orally tolerated in mice (Ikea et al., 1986) therefore making them a potential candidate for oral administration in the treatment of CRC.

1.5.2 Limitations of the $Apc^{min+/-}$ mouse model

Although the $Apc^{min+/-}$ mouse model makes an excellent candidate for the testing of potential drugs against the development of familial CRC, it does not come without its limitations. In patients with FAP, polyps tend to be isolated to the colon however in the mouse model, they grow along the intestinal track, with few to none located in the colon. Another notable difference between the animal and human form of FAP is the severity of tumours. In both mice and humans, numerous polyps develop however in the $Apc^{min+/-}$ mouse model, these adenomas are benign and rarely advance any further. In comparison, adenomas that develop in FAP patients have the potential and often to progress into invasive carcinoma (Young *et al.*, 2013)

1.6 Project hypothesis and overview

In this thesis, we hypothesized that purified sophorolipids selectively inhibit colorectal cancer cell growth *in vitro* and inhibit pre-cancerous tumour development in the $Apc^{min+/-}$ mouse model of CRC. In order to minimize batch to batch variation and the proportions

of specific congeners, SL was obtained from a tightly-controlled batch fermentation method in order to produce a stock of highly pure and well characterized LSL and ASL, that were used for all *in vitro* and *in vivo* studies.

- To characterise the cytotoxic effects of SL *in vitro*, LSL (chapter three) and ASL (chapter four) were tested at a range of concentrations on 5 different CRC cell lines representing different stages in the development of CRC. Both congeners were also tested to examine their toxic effect on normal human adherent epithelial and fibroblast cell lines.
- To test the ability of SL to mediate tumour growth, purified LSL or ASL was administered orally to $Apc^{min+/-}$ mice for 70 days (chapter three and four). In chapter five we describe the results of experiments which investigate a possible mechanism of action of ASL on disease progression *in vivo*.

Chapter 2

General Materials and Methods

2.1 Sophorolipid production and purification

The SLs used in this study were produced, purified and characterised by our collaborators (Bio base plant, Ghent, Belgium (LSL) and UPMC, Paris, France (ASL)). The isolation and purification methods are briefly described below. All SL compounds used in this thesis were derived from a single production batch.

Lactonic SL

A lactonesterase overexpressing strain of *Starmerella bombicola* (oe *sble*; (Roelants et al., 2016)) was used for the production of 96% pure lactonic diacetylated SL. HPLC-ELSD and LC-MS analysis was performed by collaborators at the Bio base plant, Ghent, Belgium and was carried out as described by (Roelants et al., 2016). This was carried out by cultivating *Starmerella bombicola* (oe *sble)* in a production medium consisting of glucose monohydrate, yeast extract, sodium citrate tribasic dihydrate, NH_4Cl, KH_2PO_4, K_2HPO_4, NaCl and $CaCl_2.2H_20$. Rapessed oil was added 48h later as a carbon source. Fermentation was carried out in 3 litre culture vessels at a temperature of 30^oC and a pH of 3.5. Fermentation was carried out for 97h before the product was removed and purified. Purification was carried out by precipitation of the ASL at 80^oC. The remaining product was filtrated using an Amicon 8200 (200 mL) stirred cell system with regenerated cellulose membranes (5, 10, and 30 kDa, filtration area 28.7 cm2; Millipore). The yeast cells were removed by centrifugation (15 min at 6000 rpm) and the cell-free fermentation medium (100 mL) was subjected to ultra- filtration. Nitrogen gas at a maximum pressure of 50 psi was applied over the filter cell. Samples were analysed using HPLC-ELSD analysis for SL content (Roelants et al., 2016).

Purified LSL was stored in a dehumidified chamber at room temperature and solubilised in 0.1% DMSO/PBS (*in vitro*) or 0.1% ethanol/ 10% sucrose (*in vivo*) for the duration of the experiments.

Acidic SL

The crude precursor used to prepare the acidic SL was purchased from Soliance (Pomacle, France; Batch # 11103A) as a lactone/acidic (~ 80/20) mixture. In order to open the lactonic form and remove the acetylated groups a classical hydrolysis step was performed in 5 M NaOH for 10 minutes. The solution was allowed to cool to RT and pH was adjusted to ~4.1. Extraction of ASL was performed as previously described (Baccile et al., 2013). In brief, following extraction, the water was removed using isopropanol under a vacuum. To extract ASL, a CH_2Cl_2/MeOH (60/40) mixture was used which prevented the remaining salt content from being extracted. After removal of the solvent *via* evaporation, the residual fatty acids were washed away using acetone (into which ASL are insoluble). This process lasted one day and it was repeated twice. The resulting white powder was allowed to dry at 55°C under vacuum for 5 days. The compound was analyzed using ^{1}H NMR and HPLC-ELSD to determine the identity and composition of the compound. The most abundant compound (75.0%) within the purified ASL is the acidic C18:1 with the remainder of the sample composed of a terminal C18:1 and C18:2 congeners (~10% each), while all other congeners are limited to less than 2%. (Baccile et al., 2012, Baccile et al., 2016).

Purified LSL was stored in a dehumidified chamber at room temperature and solubilised in PBS *(in vitro and in vivo)* for the duration of the experiments.

2.2 Cell culture

2.2.1 Cell lines

A total of five CRC cancer cell lines were chosen to determine SL bioactivity as they cover a wide range of cell types and stages of cancer development observed in the clinic (Table 2.1). The cell lines were maintained in DMEM (HT29, HT115, HCT116, LS180 or MEM (CaCo2, CCD0841-CoN, MRC5) media or supplemented with 10% foetal bovine serum (Gibco Invitrogen; Paisley, UK). All cultures were maintained at 37°C and at 5% CO2. All cell lines were maintained in a T175 flask (Thermos scientific, Paisley UK) and sub-cultured when reached 80% confluency. All cell lines used for experiments were passaged no more than 15 times and were at least 90% viable which was determined by treating the cells with 1% trypan blue solution (Invitrogen; Paisley, UK) and counting the number stained (dead) or unstained cells (alive). Viability was calculated by using the following equation: No. of Viable Cells Counted / Total Cells Counted (viable and dead) x 100 = % viable cells.

Table 2.1: Cell lines used in this thesis and their genetic characteristics

HT29	ATCC® HTB-38, Human	Colorectal adenocarcinoma (Fogh and Trempe, 1975)	Apc mutant, p53 mutant (Ying *et al*,2006)
HT115	ECACC-cultures 85061104, Human	Colorectal carcinoma (Fogh and Trempe, 1975)	Apc *wt*, p53 *wt* (Ying *et al*,2006)
HCT116	ATCC® CCL-247, Human	Colorectal carcinoma (Brattain et al., 1981)	Apc *wt*, p53 *wt* (Ying *et al*,2006)
Caco-2	ATCC® HTB-37, Human	Colorectal adenocarcinoma (Fogh and Trempe, 1975)	Apc mutant, p53 mutant (Ying *et al*,2006)
LS180	ATCC® CL-187, Human	Colorectal adenocarcinoma (Tom et al., 1976)	Apc *wt*, p53 mutant (Ying *et al*,2006)
CCD-841-CoN	ATCC® CRL-1790, Human	normal colonic epithelium (Thompson et al., 1985)	
MRC5	ATCC® CCL-171, Human	Foetal lung fibroblasts (Holliday and Tarrant, 1972)	

2.2.2 MTT assay

A total of $5x10^3$cells per well were seeded (96 well plate; Nunc Thermos scientific, UK) and allowed to attach overnight forming a monolayer. Media was then replaced with serum free DMEM (Gibco, Invitrogen; Paisley: UK) media for a further 24h. A range of final concentrations of LSL or ASL (from 0.001µg/ml – 100µg/ml) were added to the wells and the cells incubated for another 24h. Subsequently, 10µl of a 25mg/ml solution of MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide; Sigma-Aldrich Company Ltd, Dorset, UK) was added to each well and the plate was further incubated for 1h at 37°C. The remaining liquid was removed and the formazan crystals were solubilized with 100µl of DMSO (Sigma-Aldrich Company Ltd, Dorset, UK) and the absorbance at 570nm was read on a spectrophotometer plate-reader (BMG-LABTECH, Omega, Aylesbury, Bucks UK). Dosages and control groups consisted of triplicate repeats and each experiment was carried out six times, with a single representative data set (mean ± SEM) presented in the results.

2.2.3 Visualization of morphological changes induced by SL

A total of $5x10^3$ cells per well were seeded (96 well plate: Nunc Thermos scientific, UK) and allowed to attach overnight forming a monolayer. Media was then replaced with serum free DMEM (Gibco Invitrogen; Paisley, UK) media for a further 24h. A final concentration of 0, 40 or 70µg/ml of LSL or ASL was added to the cells and the cultures incubated for another 24h. The cells were subsequently imaged with a Zeiss microscope (Axio Scope 1, Zeiss, Germany). The operator was blinded to the experimental groups and three random fields were selected and captured at 20x objective magnification. Images were assessed for morphological changes by comparing each cell line to the vehicle control and looking for changes in cell shape, confluency or other morphological changes. Experiments were plated in triplicate and repeated three times, with a single representative data set shown as mean ± SEM.

2.2.4 Quantification of detached cells

A total of $5x10^3$ cells per well were seeded (96 well plate: Nunc Thermos scientific, UK) and allowed to attach overnight forming a monolayer. Media was then replaced with serum free DMEM (Gibco Invitrogen; Paisley, UK) media for a further 24h. Concentrations of 0, 50 or 100µg/ml of LSL or ASL were added to the cultures and incubated for another 24h. Supernatant (10µl) was removed from each well, placed into a 1ml microcentrifuge tube (Thermo-fisher scientific: Leicestershire: UK). Cells were stained with Syto 9 (5µM) (Invitrogen Company Ltd: Paisley: UK) and propidium iodide (5µM) (Invitrogen Company Ltd, Paisley, UK) by adding it directly into the microcentrifuge tube and incubated at room temperature for 30 minutes. The cells were centrifuged at 300x g for 5 minutes and the supernatant was removed. Cells were washed using ice-cold phosphate buffered saline (pH 7.4, Oxoid Company Limited: Leicestershire: UK) and spun onto a microscope (Thermo-fisher scientific: Leicestershire: UK) slide using a cytocentrifuge (Shandon, Thermo-fisher scientific: Leicestershire: UK) for five minutes at 1200g. Slides were subsequently imaged with a Zeiss florescence microscope (Axio Scope 1, Zeiss, Germany) at a range of objective magnifications. The operator was blinded to the experimental groups and 3 random fields were selected (40X objective magnification), with a between 1 - 250 cells counted per field of view. Following staining with Syto9 and PI, fluorescent staining was present in nucleus, with live cells appearing green while dead cells fluoresced red (Altman et al., 1993). Experiments were plated in triplicate and repeated three times, data set shown as mean ± SEM.

2.2.5 Cell viability analysis: acridine orange/ethidium bromide staining

To determine the number of necrotic or apoptotic cells resulting from addition of LSL or ASL to cultures, the cells were stained *in situ* with 10mg/ml acridine orange (Sigma-Aldrich Company Ltd, Dorset, UK) and 1mg/ml ethidium bromide (Sigma-Aldrich Company Ltd, Dorset, UK) and morphological changes were assessed by fluorescence microscopy (Ribble et al., 2005). For assessment of apoptosis, a total of $3x10^5$ cells were seeded onto a 10mm coverslip (Agar Scientific; Stansted: Essex, UK), incubated overnight and allowed to form a confluent monolayer. Following serum starvation for 24h, either LSL, ASL (at 20µg/ml or 70µg/ml, concentrations chosen based on the results from the MTT assay) or 5µM of etoposide (positive control for induction of apoptosis (Barry et al., 1993) (Sigma-Aldrich Company Ltd: Dorset: UK) was added to the wells and the cells incubated for a further 24h. To determine the number of live cells remaining on the coverslip, the samples were washed three times with ice-cold phosphate buffered saline (pH7.4, Oxoid: Leicestershire: UK), followed by incubation at room temperature in the dark, with a solution of 10µl of 1:1 acridine orange/ethidium bromide for 5 minutes and then the cells were washed 3x with ice-cold PBS and subsequently imaged with a Zeiss florescence microscope (Axio Scope 1, Zeiss, Germany) at a range of objective magnifications. The operator was blinded to the experimental groups and 3 random fields were selected and images were captured using a 40X objective). A total of 300 attached cells per coverslip were counted and identified by their morphological features as being either necrotic (red/orange nuclei), apoptotic (green condensed or fragmented nuclei) or live (green non-condensed ovoid or rounded nuclei). Experiments were plated in triplicate and repeated three times, data set shown as mean ± SEM.

2.2.6 Migration assay- Boyden chamber

A migration assay was carried out in a modified Boyden chamber, based on a previously described protocol (Chen, 2005). The individual cell lines, CCD-841-CoN, HT29 or HT115 CRC cells (10^4 cells/100μl medium), were placed in the upper compartment of a 96-well FluoroBlok transwell inserts (Analab; Lisburn, Northern Ireland) which contained 8μm pore size polyethylene terephthalate filters (Analab; Lisburn, Northern Ireland) and the cells were allowed to attach overnight. A final concentration of 0, 10 or 50μg/ml of LSL or ASL in serum free media was added to the upper chamber, while DMEM supplemented with 10% FBS was added (as a chemoattractant) to the bottom chamber and cells were left to migrate overnight in an incubator at 37°C and at 5% CO2. After the incubation, all media in the upper chamber was removed and all cells that failed to migrate was removed using a sterile cotton swab. Cells that had migrated through to the lower surface of the filter insert were stained with a 0.1% solution of crystal violet (Sigma-Aldrich Company Ltd: Dorset: UK) made up in 25% methanol (Sigma-Aldrich Company Ltd: Dorset: UK). A total of three random areas were chosen and a minimum of 1 cell and a maximum of 300 cells stained with crystal violet was counted under a light microscope at 40x (Zeiss microscope; Axio Scope 1, Zeiss, Germany). Migration rates was expressed as total percentage of the control. Experiments were plated in triplicate and repeated three times, data set shown as mean ± SEM.

2.2.7 Invasion assay

This assay is an adaption to the migration assay using a Boyden chamber as previously detailed (Albini et al., 1987). The upper filter insert compartment of a 96-well FluoroBlok transwell boyden chamber (Analab; Lisburn, Northern Ireland) containing 8μm polyethylene terephthalate pore inserts (Analab; Lisburn, Northern Ireland) were removed and coated with 10μg ECM (Sigma-Aldrich Company Ltd, Dorset,-Aldrich; UK) at room temperature for 1hr. CCD-841-CoN, HT29 or HT115 CRC cells (10^2 cells/100μl medium) were placed on top and treated with 0, 10 or 50μg/ml of LSL or ASL in serum free media.

DMEM supplemented with 10% FBS was used as a chemoattractant in the bottom chamber and cells were left to migrate through the ECM and through the insert following overnight incubation at 37°C and with 5% CO_2. After the overnight incubation, all media in the upper chamber was removed and cells that failed to migrate were removed using a sterile cotton swab. Cells that had migrated through to the lower surface of the filter insert were stained with a 0.1% solution of crystal violet (Sigma-Aldrich Company Ltd: Dorset: UK) made up in 25% methanol (Sigma-Aldrich Company Ltd: Dorset: UK). A total of three random areas were chosen and a minimum of 1 cell and a maximum of 300 cells stained with crystal violet were counted with a light microscope using a 40x objective (Zeiss microscope; Axio Scope 1, Zeiss, Germany). Migration rates was expressed as total percentage of the control. Experiments were plated in triplicate and repeated three times, data set shown as mean ± SEM.

2.2.8 Scratch assay

For the wound healing scratch assay (Todaro et al., 1965, Rodriguez et al., 2005), $1.6x10^5$ CCD-841-CoN, HT29 and HT115 cells were plated in each well of a 6 well plate (Nunc Thermos scientific, UK) and allowed to attach overnight. A "wound" was made in the confluent monolayer by scratching a line in the centre of the well using a sterile toothpick. Cells were rinsed very gently with PBS three times and serum free media supplemented with vehicle-control or 10µg/ml ASL was added to the cultures before incubation for another 72 hours. Images were captured at 10x objective magnification at various time points using a Nikon microscope (Nikon ELWD TI SCP, Japan) and stored as .tif files for offline analysis. The area of the gap not occupied by cells was measured using ImageJ software (Schneider et al., 2012). After 72h, measures were taken from the area not occupied by cells. Experiments were plated in triplicate and repeated three times.

2.3 Animal model

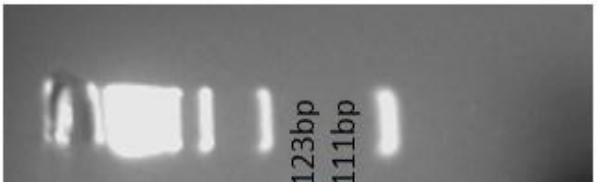
All animal procedures were approved by the animal care and ethics committee (Ulster University) and national (UK Home Office) ethical guidelines.

The $Apc^{min+/-}$ male and wild type female mice were housed together for breeding purposes and subjected to a 12/12 light cycle. Food and water were available *ad libitum* and animals were weighed on a weekly basis to evaluate consumption. Mice were monitored on a daily basis by recording grooming habits, behaviour, activity levels and general well-being (for details see Appendix 1). Once treatment with SL commenced, a loss of 10% or more of body weight and/or any obvious signs distress resulted in immediately euthanization by an overdose of inhaled CO_2.

2.3.1 Genotyping

Ear punch biopsies were obtained from the 21-day old progeny of $Apc^{min+/-}$/*wt* crosses for the purpose of genotyping, using primers specific for the APC mutation . Genomic DNA was isolated from samples of ear tissue by first solubilizing them in 75µl of an alkaline lysis reagent (25mM NaOH, 0.2MmM disodium EDTA; (Sigma-Aldrich Company Ltd, Dorset, UK) in a 0.2ml tube (Thermo-Fisher scientific: Leicestershire: UK) at 95°C for 40 minutes, allowing them to cool and neutralizing the digested sample with 75µl of Tris-HCl (40mM; (Sigma-Aldrich Company Ltd, Dorset, UK). The digested samples were mixed with PCR master mix (Qiagen Company Ltd, Manchester, UK), *taq* polymerase (Qiagen Company Ltd; Manchester: UK), nuclease free water (Qiagen Company Ltd; Manchester: UK), and Apc^{min+} specific primers (forward: TCT CGT TCT GAG AAA GAC AGA AGC T/reverse: TGA TAC TTC TTC CAA AGC TTT GGC TAT;100µm each; Invitrogen Company Ltd, Paisley, UK). Samples were placed in a thermocycler (Techne TC-5000 Gradient Thermocycler, Hanwell, London UK) and a PCR reaction performed under the following conditions: 94°C, 2min; (94°C, 1min; 60°C, 1min; 72°C, 1min) for 30 cycles followed by a final extension step at 72°C for 2 minutes. The PCR

products were subjected to *Hind*III digestion (Invitrogen, Paisley, UK) for 1h at 37°C followed by a 20 minute denaturing step at 65°C. Digested PCR samples were electrophoresed on a 4% agarose/TBE-buffered gel (Sigma-Aldrich, Dorset, UK) for 40 minutes. The presence of a single band at 111bp indicated a *wt* mouse, while an additional band at 123bp indicated a heterozygous $Apc^{min+/-}$ mouse (Fig. 2.1).



e band in-
$Apc^{min+/-}$

2.3.2 Sophorolipid dosing

Mice were treated orally *via* a voluntary drinking routine (by dispensing solutions through a sterile p20 pipette tip) every other day with either vehicle-only control (LSL; 0.1% ethanol/ 10% sucrose) (ASL; 10% sucrose in saline) or a solution containing 50mg/kg body weight of LSL suspended in vehicle. Mice were observed for 20 minutes following feeding treatment to ensure all solution was ingested.

2.3.3 Tolerance study

To test the ability of *wt* and $Apc^{min+/-}$ mice to tolerate the oral administration of SL, a pilot study was undertaken in C57 *wt* mice of which 2 mice/group were fed a solution of either LSL or ASL (0.5, 5 or 50mg/kg) for 5 weeks. This determined that there were no adverse effects to as body weight or general well-being observed. During the experiment, body weights, food and water consumption were measured weekly. After 5 weeks of treatment, the mice were euthanized by an overdose of CO_2 inhalation. Subsequently, major organs (liver, stomach, kidneys, lungs, heart, spleen and pancreas) were isolated, weighed and changes in gross morphology such as shape and colour were recorded.**2.3.4 Efficacy study**

At five weeks of age, *wt* and $Apc^{min+/-}$ males and females (separated by gender), were placed into experimental groups (10 per group). Both *wt* and $Apc^{min+/-}$ mice were fed vehicle or 50mg/kg LSL or ASL for 10 weeks. During the experiment body weights, food and water consumption was measured every other day. At the mid-point of the SL treatment protocol (35 days), blood was extracted from the tail vein from the mice for haematocrit analysis. This was carried out by first dipping the mouse's tail in warm water (40ºC) to expose the veins. Blood was collected from the vein using a 23G needle and collected in EDTA tubes (Aquilant Scientific, Down, NI). After 70 days of treatment, mice were euthanized with an overdose of non-recovery anaesthesia (Ketamine (100 mg/kg BW) (Ketavet; Pfizer, Berlin, Germany) and xylazine (16 mg/kg BW) (Rompun;

Bayer, Leverkusen, Germany) and blood was immediately collected by cardiac puncture, placed into EDTA tubes (Aquilant Scientific, Down, NI) and the haematocrit determined (Cole-parmer, Trickenham,UK) within 60 minutes of extraction.

2.3.5 Tissue collection and assessment

Following euthanasia, samples of intestinal tract, colon, spleen, heart, liver, kidneys and lungs were carefully removed, weighed and then fixed in 10% buffered formal saline (pH7.4, Oxoid: Leicestershire: UK). The intestinal tracts were laid out (Fig 2.2) and divided into the duodenum, jejunum and ileum according to the description of (Casteleyn et al., 2010) and inspected for the appearance of Peyer's Patches along the outside of the intestinal tract. After identification of the specific intestinal regions, the samples were cut longitudinally, the number of polyps was recorded and their diameters measured with callipers (Fig 2.2). Individual specimens were then cut into ~2cm strips and placed in cassettes containing 10% BFS, prior to standard tissue processing and wax embedding.

2.3.6 Tissue processing and wax embedding

Cassettes were subsequently placed into 10% neutral buffered-formalin (Sigma-Aldrich; Dorset, UK) for 1hr and placed into the Leica TP 1020 tissue processor (Leica; Milton Keynes, UK). The tissue was processed according to the following automated machine program: 70% ethanol (2h), 80% ethanol (2h), 90% ethanol (2h) 100% ethanol (1h), 100% ethanol (1h) 100% ethanol (1h), xylene (1h 30min), xylene (1h 30min), paraffin wax (4hr), paraffin wax (2hr). Following the wax infiltration, the tissue was embedded by pouring melted wax into a medium surgipath embedding mould (Leica; Milton Keynes, UK) and critically orientating the tissue.

ssue.

t flat for orientation. b)

2.3.6 H&E staining

To assess qualitative histopathological changes in the intestines and spleen, the paraffin –embedded tissues were cut using a microtome (Shandon; Cheshire, UK) into 5µm sections. The sections were then placed on glass slides, cleared with xylene, dehydrated in descending grades of ethanol, stained with Mayer's haematoxylin and eosin stain (Sigma-Aldrich, Dorset, UK) and examined with a Zeiss light microscope (Axio Scope 1, Zeiss, Germany) at a range of objective magnifications.

2.4 Statistical analysis

Statistical analyses of *in vitro* data was performed using either one-way ANOVA or student's t- test using the GraphPad Prism programme (GraphPad software, San Diego, USA). All comparisons between *in vivo* groups were assessed using a students' t-test. A value of $p < 0.05$ was defined as being statistically significant. Significance is signified as follows in all Figures: * $p<0.01$, ** $p<0.001$ ***$p<0.0001$.

Chapter 3

Lactonic Sophorolipids increase tumour burden in $Apc^{min+/-}$ mice.

The majority of data obtained from this chapter is included in a publication in PLoS one (Callaghan et al., 2016). This manuscript is included as Appendix 3.

3.1 Introduction

SL produced by the non-pathogenic yeast, *Candida bombicola* has received an increase in research interest due to their reported biological functions. This has prompted the exploration and possible manipulation of these biodegradable SL for their potential clinical and pharmacological applications.

SL are created *via* fermentation of yeast producing a compound made up of LSL or ASL mixtures. LSL and ASL differ in chemical structure and thus may have differing potential applications. For instance, LSL have shown to be the preferred SL compared to ASL and other derivative congeners as it excels as an antibacterial, anti-viral and anti-inflammatory agent. LSL concentrations as low as 0.01% w/v, impart lower surface tension to solutions and are proving useful in detergent formulations (Cox et al., 2013). LSL detergents have been shown to be more effective at stain removal at lower washing temperatures making them more economical and environmental friendly in comparison to other commercially available detergents (Marchant and Banat, 2012).

LSL have an established reputation of being strongly anti-microbial, with a pronounced effect against gram-positive bacteria *in vitro (Shah et al., 2007)* and in sepsis models *in vivo (Bluth et al., 2006).* They have also shown demonstrated anti-fungal activity against plant pathogens such as *Phytophthora sp.* and *Pythium sp.* (Schofield et al., 2012) as well as having spermicidal activity. However more recent *in vitro* studies indicate that LSL may have potential as anti-cancer agents.

The *in vitro* activities of SL has been reviewed, with LSL enriched preparations showing potent cytotoxicity against human liver (HT402), lung (A549) and leukemic (HL60 &

K562) cells (Chen et al., 2006a). Cruder preparations of SL are reported to show dose-dependent, anti-proliferative and pro- apoptotic activity against pancreatic cancer (H7402, HPAC) (Fu et al., 2008) and esophageal cancer (KYSE450, KYSE109) cell lines (Shao et al., 2012).

Conclusions on the specificity of such diverse SL preparations (i.e. many are poorly characterized and contain unspecified mixtures of LSL and ASL) to transformed cells growth is complicated by the inappropriate use of normal cell controls to properly assess their specific toxicity. One such study used non-adherent peripheral blood mononuclear cells (PBMCs) (Fu et al., 2008) to test the compounds toxicity in comparison to pancreatic cancer cells. This is not an appropriate control as it is a non-adherent cell line and does not originate from the same organ as the cancer being tested.

The purity and composition of SL preparations used in these bioassays is highly variable, with most studies not disclosing the molecular species or their relative abundances within the "purified" mixtures (Fu et al., 2008, Chen et al., 2006b). Acetylated LSL predominate in most SL mixtures (characteristically 70 – 85%) (Hu and Ju, 2001), therefore, due to the high percentage of LSL, studies claim these formulations as being in "purified" forms.

Despite a number of studies showing that SL preparations have anti-proliferative effects on tumour cell lines *in vitro*, there are few *in vivo* studies in tumour models. To our knowledge, the only other study reported the effects of SL mixture in a cervical cancer tumour xenograft model. Here Li et al, demonstrated that intragastrically SL administered for 12 days resulted in a dose dependent (5, 50 and 500mg/kg) decrease in tumour weights in BALB/c nude mice injected with HeLa cells (Li et al., 2016a).

In order to minimize batch to batch variation and reduce specific congeners (2.5% ASL and <1% free fatty acids as a proportion of dry weight) we used stock samples from our collaborators who used a tightly-controlled batch fermentation method of 96% pure and well characterized LSL that was used in all our *in vitro* and *in vivo* studies.

In test the potential toxicity of purified LSL *in vitro*, we examined its' effect on 4-5 CRC cells lines which cover the different severities of CRC seen in humans. Two normal adherent and well known cell lines – a colonic epithelial coupled with a lung fibroblast cell line was also treated with various concentrations of LSL was used to examine the ability of SL to differentiate between normal and cancerous cells *in vitro.*

To further quantify the effects in *vivo*- LSL was orally administered to the $Apc^{min+/-}$ mice, a gold standard CRC model use to test chemotherapeutic agents. Prior the commencement of the study, a "pilot" study was carried out to ensure that both C57 and $Apc^{min+/-}$ mice could tolerate the oral administration of the LSL at doses of up to 50mg/kg.

3.2 Hypothesis:

Purified forms of LSL specifically inhibit colorectal tumour cell growth *in vitro* and polyp growth in a FAP model *in vivo*.

Aims and objectives:

1) Obtain a purified and well characterized LSL stock produced by *Starmerella bombicola.*

2) Assess the cytotoxic properties of a purified LSL sample on four unrelated colorectal cancer cell lines: HT29, HT115, HCT116, Caco-2 and in addition to two non-transformed lines: normal human colonic epithelium CCD-841 CoN and lung fibroblast MRC5 *in vitro.*

3) Determine the ability of orally administered LSL to inhibit polypgrowth in $Apc^{min+/-}$ mice, a well-established model of FAP.

3.3 Results

Production and purification of LSL

The production of the C18:1 lactonic diacetylated SL (Fig 3.1) used for our *in vitro* and *in vivo* studies was produced by collaborators (Roelants et al., 2016). A *S. bombicola* oe *sble* strain was employed and a bioreactor experiment similar to one previously described (Roelants et al., 2016) was carried out. However, instead of rapeseed oil, oleic acid was used as the hydrophobic carbon source. This resulted in a very uniform SL product (Fig 3.2) containing 99% SL (97.3 % C18:1 SL [M_w = 688], 1.3 % C18:2 SL and 0.4 % C18:0 SL).

The final purity of the sample was >99.5% SL and the composition in terms of SL was determined to be 96% diacetylated C18:1 LSL (M_w = 688; Fig. 3.2, 3.8 % diacetylated C18:1 ASL (M_w = 706; Fig. 3.2) and minor impurities consisting of 0.04% free fatty acids/oil, 0.001% glucose, 0.004% glycerol and total nitrogen of 0.14%. This batch sample was stored at room temperature and solubilized in 0.1% DMSO/PBS (*in vitro*) or 0.1% ethanol/ 10% sucrose (*in vivo*) for use in all the experiments described in this chapter.

Figure 3.1: Lactonic Sophorolipid (LSL)

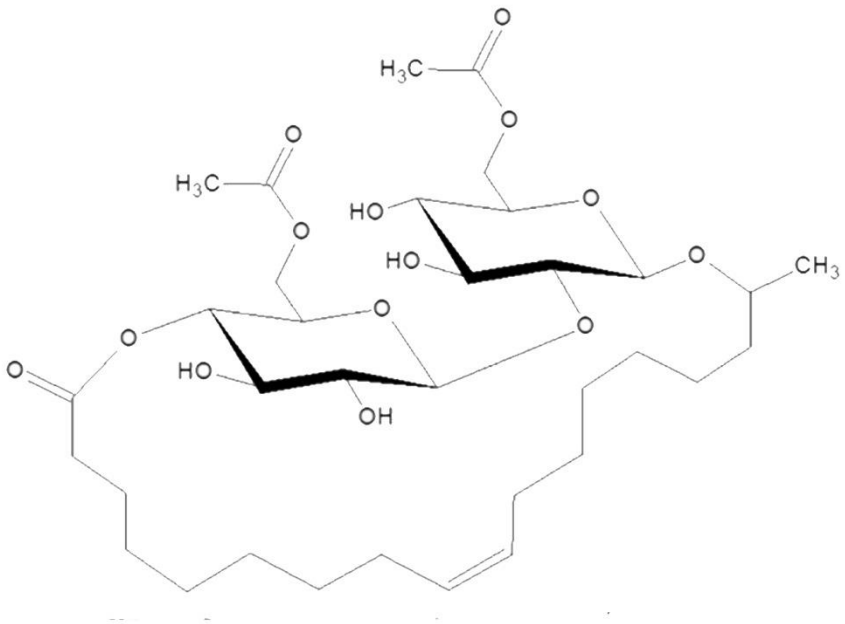


Fig 3.1: The structure of C18:1 diacetylated LSL.

Figure 3.2: Purification of LSL produced by *S. bombicola.*

Fig 3.2: HPLC-ELSD and LC-MS analysis of the LSL precipitate from the bioreactor. (a) The MW 688 g/mol corresponds to diacetyl C18:1 LSL. (b) HPLC-ELSD analysis following purification of the LSL precipitate. The MW 706 g/mol = diacetyl C18:1 ASL, which is the result of minor hydrolysis of the MW 688 compound.

LSL reduces cell viability in CRC cells

LSL concentrations above 20µg/ml resulted in reduced viability of both colonic epithelial (CCD-841-CoN) and lung fibroblast (MRC5) cell lines (Fig. 3.3a; $p<0.0001$), in addition to Caco2, HCT116 and LS180 colorectal tumour cell lines (Fig 3.3b; $p < 0.05$). HT29 cells show a trend towards an increase in viability at doses between 20 - 40µg/ml; however, this is not statistically significant ($p > 0.05$). In both HT29 and HT115 colorectal cancer cell lines a significant decrease in viability was observed at doses exceeding 70µg/ml (Fig. 3.3b; $p < 0.001$).

LSL induces morphological changes and loss of adherence in vitro

Microscopic examination of confluent cultures of CCD- 841-CoN cells revealed a bimodal morphology following exposure to vehicle, whereas at doses of 40 and 70 µg/ml LSL there were large areas devoid of cells, with remaining adherent cells displaying a shrunken and rounded phenotype (Fig. 3.4). In vehicle-treated cultures, all the cancer cell lines (HT29, HT115, HCT116 and Caco2 cells) display densely packed, cobblestone-like monolayer morphology. HT29 and HY115 cells had no obvious change in phenotype at a dose of 40µg/ml LSL. At a dose of 40 µg/ml, LSL resulted in the detachment of HCT116 and Caco2 cells (Fig. 3.4). In HT29, HT115, HCT116 and Caco2 cells that were exposed to 70µg/ml LSL, the confluent monolayer was disturbed and there were clear signs of cell rounding and cell-free areas indicative of detachment (Fig. 3.4).

Following treatment with the middle and maximum doses - 0, 50 and 100µg/ml LSL, detached cells were isolated from the supernatant of wells to determine if they were alive or dead, using propidium iodide and Syto 9 staining (Fig 3.5). At doses of 50 and 100µg/ml, all cells found in the normal CCD-841-CoN supernatant were stained red, positive for cell death (Fig 3.5a $p<0.001$). In the cancer cell lines, 4% of cells found in the supernatant stained green – a positive for being alive while 96% of the detached cells were dead (Fig 3.5b-e $p<0.01$). At 100µg/ml, all detached colorectal cancer cells were dead ($p<0.001$)

Figure 3.4: LSL results in morphological changes in CRC cells *in vitro*.

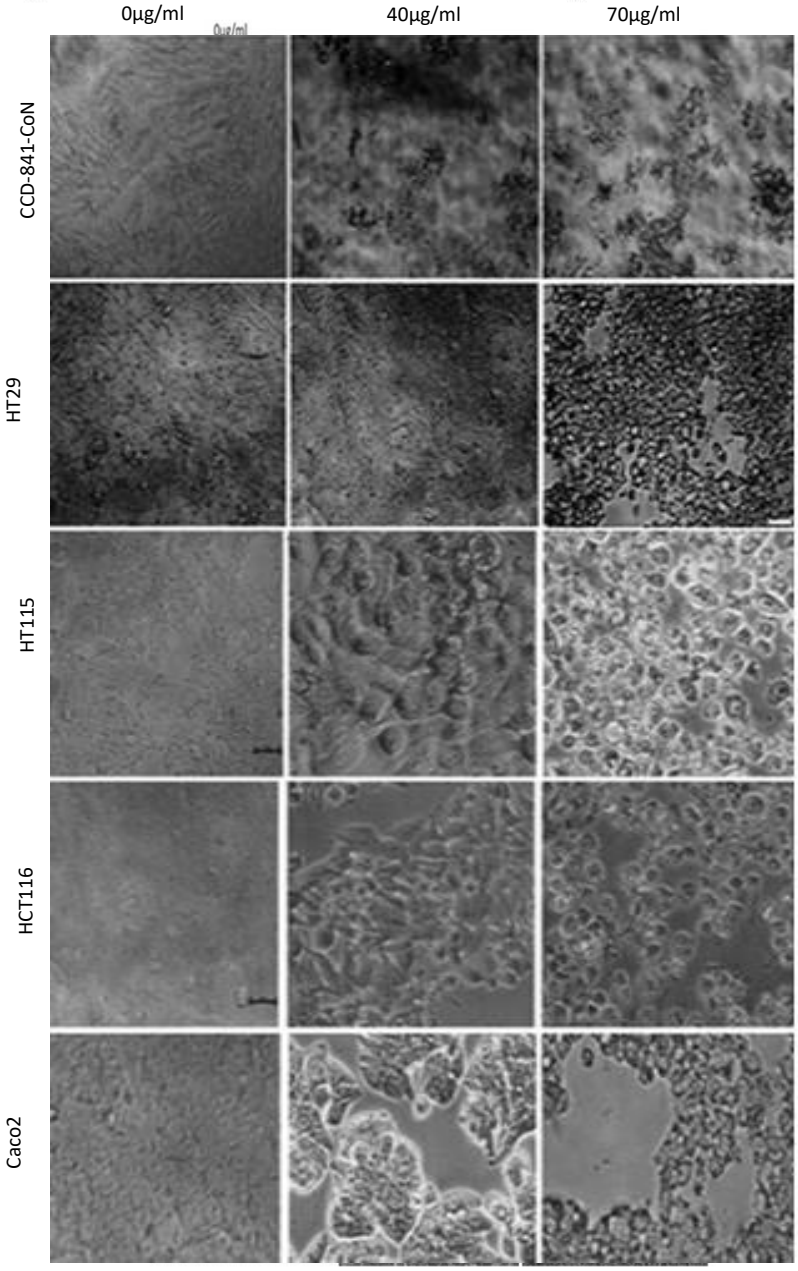


Fig 3.4: Light micrographs from normal colonic epithelial and CRC cell lines. CCD-841-CoN cells treated with vehicle-only control show a typical bipolar morphology. After treatment with 40µg/ml (central) or 70µg/ml LSL (right) the remaining adherent cells are rounded and there are large regions devoid of cells. In CRC cells, HT29, HT115, HCT116 and Caco2 treated with vehicle-only, the cells are densely packed with a cobblestone-like morphology. Following treatment with 40µg/ml LSL (middle), HT29 and HT115 show are no discernable changes in c ll morphology, however treatment with 70µg/ml LSL (right) leads to cell rounding and conspicuous cell free areas. Treatment of 40µg/ml LSL in HCT116 nad Caco2 cells resulted in disruption to the monolayer. At doses of 70µg/ml LSL, cellular rounding and cell free areas were observed. Scale bar = 50µm for each image

Figure 3.5: LSL treatment results in the detachment of dead cells

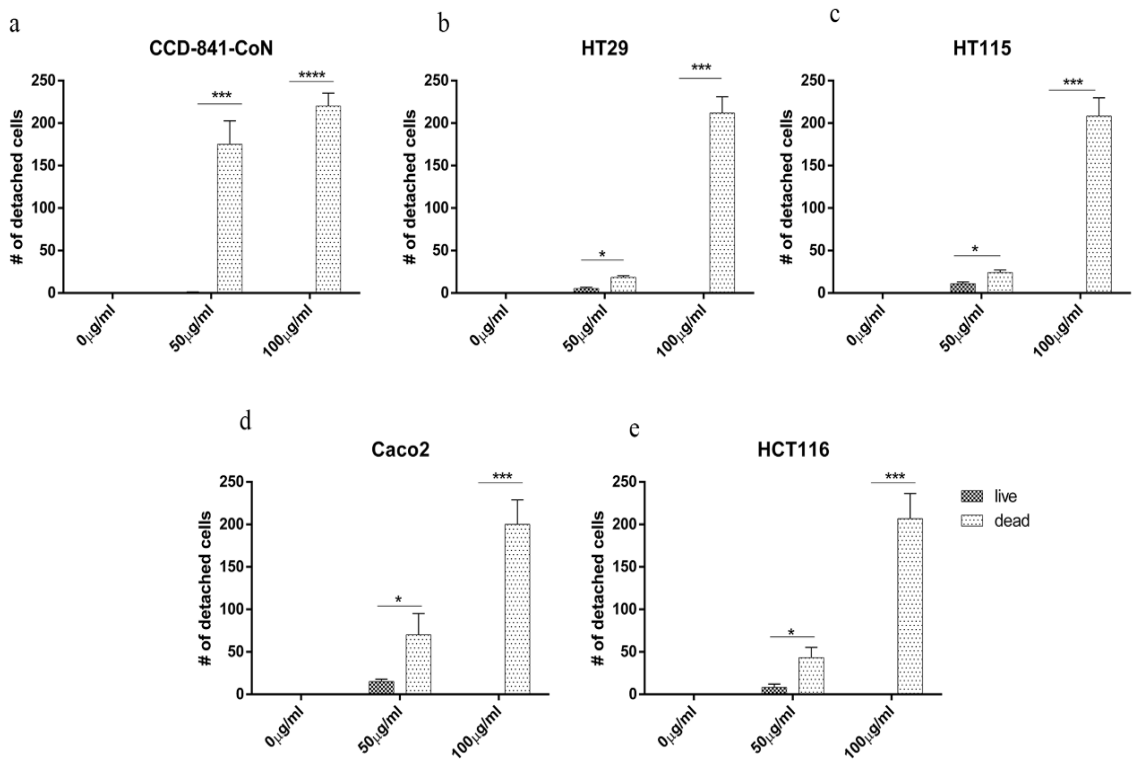


Fig 3.5: LSL treatment results in the detachment of dead cells. Quantification of live or dead cells using propidium iodide and Syto 9 staining on cells extracted from the supernatant of cultures treated with 0, 50 or 100µg/ml of LSL. Following treatment with 50µg/ml LSL, CCD-84l CoN (a), all detached cells in the supernatant were dead (*** p <0.001). At the same concentration, a small number of colorectal cancer cells (b-e) were alive (4%) while the remainder were dead (96% *p< 0.01). In CCD-84l-CoN control cultures and colorectal cancer cells exposed to 100µg/ml LSL, 100% of cells counted in the supernatant were dead (*** p <0.0001). Graphs representative of mean ± SEM. Significance was calculated using a student's t-test and one-way ANOVA.

LSL induces cell death *in vitro*

Cells treated with LSL were assessed *via* ethidium bromide staining and morphological analysis to determine if they were alive, apoptotic or necrotic as outlined in Chapter 2.2.5. LSL treatment resulted in a higher proportion of cells undergoing necrosis compared to apoptosis in both normal colonic as well as the four colorectal cancer cell lines we examined (Fig. 3.6). In CCD-841-CoN cultures a dose of 20µg/ml LSL resulted in ~70% cell death (Fig. 3.6f), the majority of which showed morphological features consistent with necrosis (Fig. 3.6p). Both HCT116 (Fig. 3.6i $p<0.001$) and Caco2 (Fig. 3.6j $p<0.001$) were susceptible to cell death at a dose of 20µg/ml LSL, while HT29 (Fig. 3.6g) and HT115 (Fig. 3.6h) were relatively resistant. In CCD-841-CoN cells exposed to 70µg/ml LSL, the few attached cells available for quantification (Fig. 3.6k) were either necrotic or apoptotic whereas in HT29 (Fig. 3.6l), HT115 (Fig. 3.6m), HCT116 (Fig. 3.6n) and Caco2 (Fig. 3.6o) all showed a significant increase in both necrotic ($p < 0.0001$) as well as apoptotic ($p < 0.001$) cells when compared with vehicle only (Fig. 3.6a-e). Etoposide was used as a positive cell death inducer control (Fig 3.6p-t). Etoposide induced primarily apoptosis in all cell lines with the exception of Caco2 where higher levels of necrosis was observed.

Figure 3.6: LSL induced both necrosis and apoptosis *in vitro*.

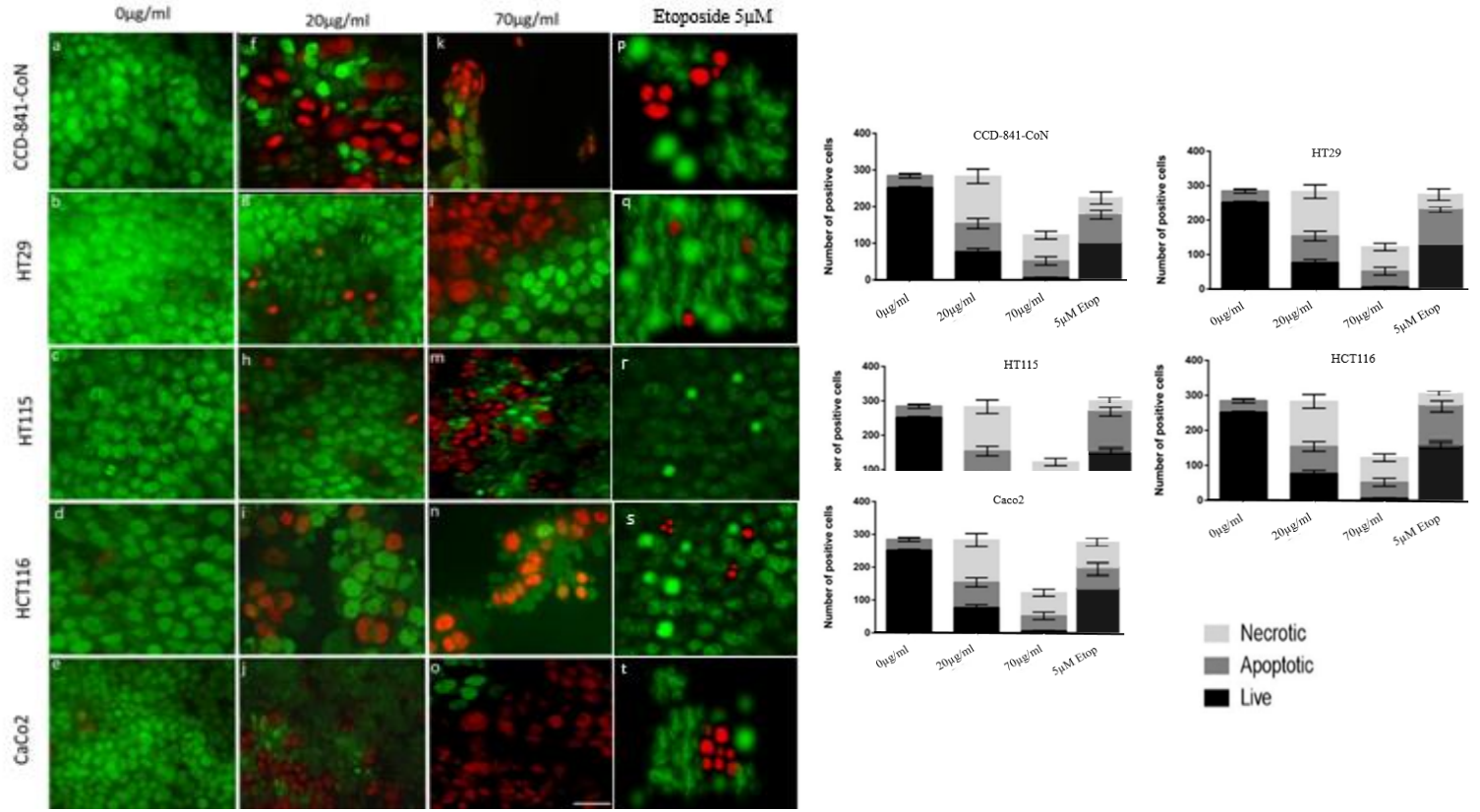


Fig 3.6: Photomicrographs of acridine orange and ethidium bromide stained cultures following treatments with 0 (a-e), 20 (f-j) or 70µg/ml (k-o) LSL or 5 µM etoposide (p –t) for 24hrs and quantification of live, apoptotic or necrotic cells. The vast number of CCD-841-CoN(a) and cancer cells treated with vehicle control are morphologically viable with a small number showing condensed nuclei (apoptotic). 20µg/ml LSL resulted in necrosis (red/orange clusters) in all cell lines, although CCD-841-CoN (k.p ****p<0.0001) and Caco2 cells (j.o ** p<0.001) were more susceptible. At 70µg/ml, very few adherent CCD-841-CoN cells were observed (k), remaining adhered cells were either necrotic or apoptotic (p). HT29 (l), HT115 (m), HCT116 (n) and Caco2 (o) cells exposed to 70µg/ml LSL all had 50% of cells with morphological features of cells death and statistically significant increases in either the numbers of necrotic (*** p<0.0001) or apoptotic cells (**p<0.001) as compared with vehicle only controls.

To determine the effects of LSL on cell migration and invasion, the Boyden chamber assay was used as described in chapter 2.2.6. CCD-841-CoN treated with the vehicle-only control solutions were set to a 100% migration rate to compare with the LSL treatments. In media supplemented with 10µg/ml LSL cell migration was reduced to 32% of control values ($p < 0.0001$ Fig 3.7a). The addition of 50µg/ml of LSL further decreased migration to 11% of control values ($p < 0.0001$ Fig 3.7a). Concentrations of 10µg/ml did not significantly alter the migration of either HT29 or HT115 cultures. However, at 50µg/ml the migration of HT29 and HT115 decreased to 60% ($p < 0.001$ Fig 3.7a) and 73% ($p < 0.001$ Fig 3.7a) respectively of control values for these cell lines.

To determine the ability of LSL to influence the invasive properties of cancer cells, CCD-841-CoN, HT29 or HT115 cells were plated on a layer of Matrigel™ in the upper part of a Boyden chamber. The normal CCD-841-CoN cell line failed to invade through the Matrigel. At 10µg/ml there was no significant difference in invasion percentages in the HT29 or HT115 cells compared to vehicle control. At 50µg/ml LSL, the percentage of invasion in HT29 and HT115 cells was reduced to 30% ($p < 0.001$ Fig 3.7b) and 50% ($p < 0.0001$ Fig 3.7) of control values respectively.

Figure 3.7: LSL reduces migration and invasion of CRC cells at high concentrations

Fig 3.7: a – Boyden chamber migration assay of normal and CRC cells treated with 10 or 50ug/ml LSL. Migration was scored as a percentage compared to the vehicle control. Following treatment of 10ug/ml LSL, migration rates decreased to 32% (*** $p < 0.0001$) in CCD-841-CoN, however there were no significant decrease in migration in the CRC cell lines HT29 and HT115. At 70ug/ml, only 11% of CCD-841-CoN migrated through the chamber (*** $p < 0.0001$) while 60% (** $p \leq 0.001$) and 73% (** $p < 0.001$) of HT29 and HT115 migrated through. b – Matrigel invasion assay normal and CRC cells treated with 10 or 50ug/ml LSL. Invasion was scored as a percentage compared to the vehicle control. Both vehicle and treated CCD-841-CoN cells failed to invade through the matrix. At 10ug/ml there were no significant difference in the invasion percentage in HT29 and HT115 cells ($p < 0.01$, $p < 0.12$ respectively). Doses of 70ug/ml resulted in 30% total invasion in HT29 (** $p < 0.001$) and 50% in HT115 (*** $p < 0.0001$). Statistical analysis determined using students t-test. Values represented by mean ± SEM. n=4 with 3 independent repeats.

***wt* and Apc$^{min+/-}$ mice tolerate the oral administration of LSL**

In order to determine the palatability and assess any potential contra-indication for oral administration of LSL, a small number (3 per LSL concentration in a dose escalation protocol) of *wt* and Apc$^{min+/-}$ mice were fed a vehicle consisting of either 0.1mM DMSO in 10% sucrose water or 0.5, 5 or 50mg/kg LSL dissolved in 0.1 DMSO /10% sucrose water for 5 weeks.

The weights (Fig 3.8a) of the mice and their water (Fig 3.8b) and food consumption (Fig 3.8c) was measured each week. Both *wt* and Apc$^{min+/-}$ mice gained weight at the same rate which was not affected by LSL administration. On average, both vehicle and LSL treated *wt* and Apc$^{min+/-}$ consumed 12.5 grams of food and 13.4 grams of water per week.

Mice were also monitored for any change to normal behavioral habits such as grooming, nesting and reaction to humans during the study. There was no change in behavioral activity noted across the different treatment groups or LSL dosing concentrations (Appendix 1).

On completion of the tolerance study, major organs (liver, stomach, kidneys, lungs, heart, spleen and pancreas) were removed weighed and a gross inspection was carried out (Fig 3.9b). There were no significant differences in organ weights of the vehicle treated compared to LSL treated mice. There were no statistically significant differences in weight gain, food or water consumption, behavioral activity or organ weights in either *wt* or *Apc$^{min+/-}$* mice at any of the concentrations of LSL (0.5, 5, 50mg/kg) tested (see Appendix 1).

Figure 3.8: LSL are tolerated by *wt* and Apc$^{min+/-}$ mice

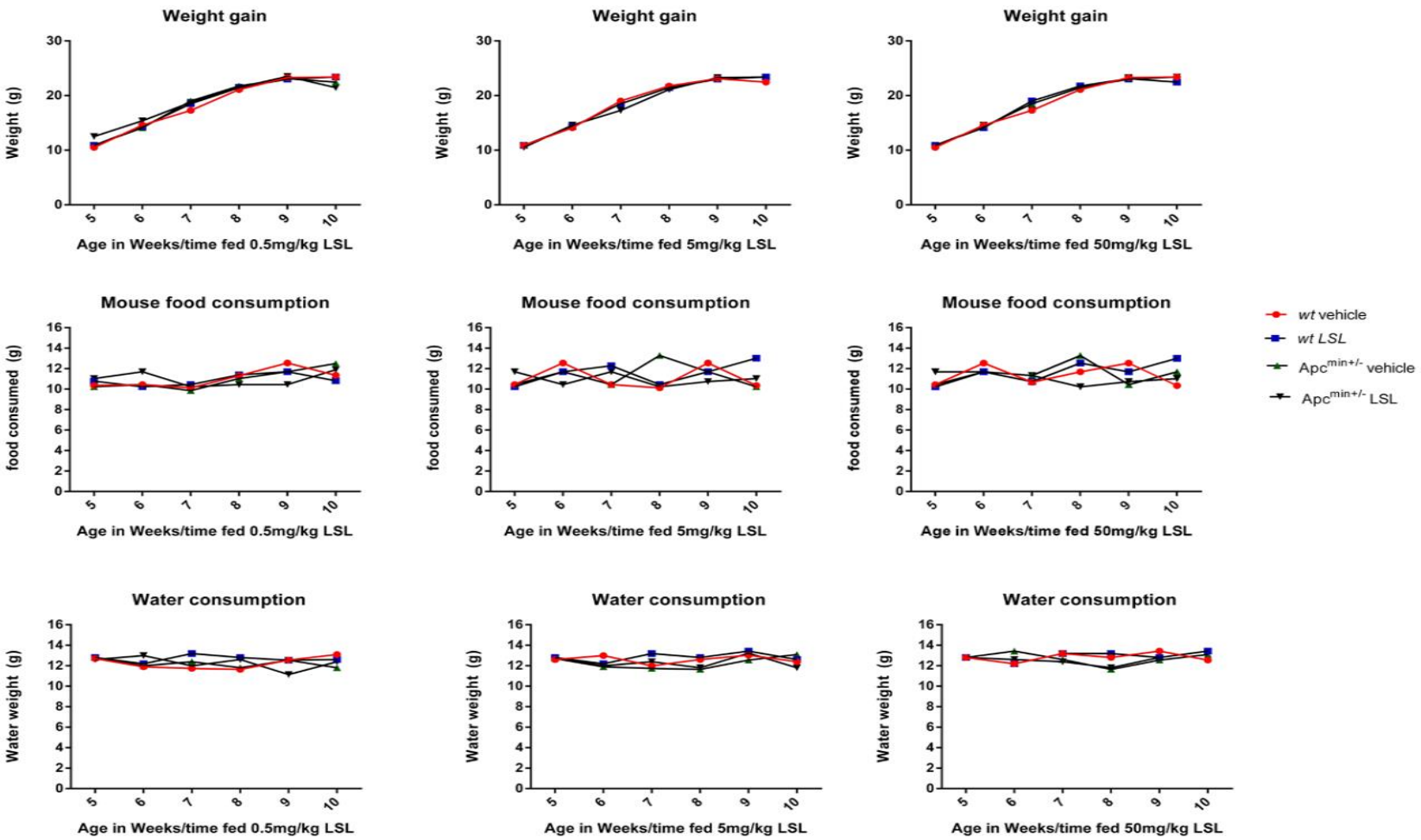
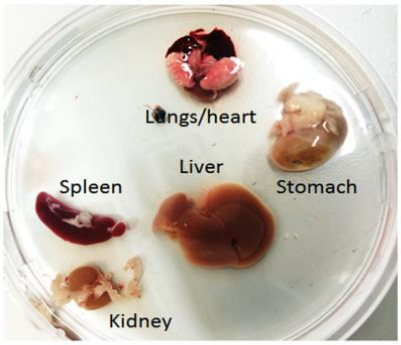


Fig 3.8: *wr* and Apc$^{min+/-}$ mice were fed vehicle or 0.5, 5 or 50 mg/kg of LSL every other day for 5 weeks and monitored closely. Mouse weights, food and water consumption was measured on a weekly basis. There were no signifcant differences in weight (a), food consumption (b) or water consumption (c) . Statistcal analysis was carried out using one-way ANOVA. Values represented by mean. n=6 per group.

Figure 3.9: Short term LSL treatment exerts no adverse effects on organ apperance or weight.

a



b

	wt vehicle	*wt LSL*	Apc$^{min+/-}$ *vehicle*	Apc$^{min+/-}$ LSL
Liver	0.751g	0.755g	0.750g	0.749g
Stomach	0.521g	0.532g	0.515g	0.525g
Kidneys (both)	0.412g	0.412	0.410g	0.413g
Lungs	0.11g	0.10g	0.12g	0.12g
Heart	0.12g	0.11g	0.11g	0.10g
Spleen	0.15g	0.16g	0.545g	0.555g
Pancreas	0.198g	0.199g	0.197g	0.198

Fig 3.9: wt or Apcmin+/- mice were fed vehicle or 0.5, 5, 50mg/kg LSL for 5 weeks. There were no changes in the liver, stomach, kidneys, lungs, spleen or pancreas morphology (a) or weights (b) between treatment groups. a; Organs was taken from 50mg/kg but representative of all three LSL concentrations.

Oral administration of LSL to $Apc^{min+/-}$ mice exacerbates polyp development

Genotyping of mice was undertaken following genomic DNA extraction, PCR and subsequent restriction enzyme digestion and electrophoresis; this methodology yielded a single 111bp band for the *wt* allele or dual 111/123 bp alleles (Fig. 3.10a) that are consistent with a heterozygous $Apc^{min+/-}$ mouse. On the basis of genotyping, mice were randomly assigned to either LSL or vehicle-only dosing groups, irrespective of gender. The weights of both *wt* and $Apc^{min+/-}$ mice fed with either vehicle-only control or LSL solutions were not significantly different (25.2g vs 24.9g; NS $p < 0.1$) and there were no differences in water (98.2ml vs 99ml; NS, $p > 0.05$) or food (180.7g vs 178g; NS $p > 0.05$) consumption over the duration of the experiment.

After two weeks of treatment, bloody mucus and small polyps (0.3mm +/- 2) were observed around the anus of the LSL treated mice (n=6/10 mice). By the end of the LSL treatments the anal polyps were 1-2mm in size and there were obvious signs of bleeding from the intestinal tract (3.10b).

Following euthanasia and removal and inspection of the intestinal tract, this revealed an exacerbation in the growth of polyps in LSL treated $Apc^{min+/-}$ mice compared to the vehicle-control mice (Fig 3.11). The gross morphological appearance of unfixed flat mounted ilea from *wt* mice (Fig. 3.11 top) treated with vehicle-only (left) or 50 mg/kg LSL (right) was characterized by a flattened, uniformly smooth mucous epithelium. In vehicle-only treated $Apc^{min+/-}$ mice (Fig. 3.11;), there was clear evidence of occult

bleeding throughout the ileal segment and numerous polyps (modal diameter 2mm; Fig. 3.11) compared to *wt* mice. Following treatment with 50mg/kg LSL for 70 days, there is clear evidence of recent bleeding as well as a greater number (vehicle-only = 55.5 ± 3.3 *vs* 50mg/kg LSL = 70.5 ± 7.8; Fig. 5c; $p < 0.05$) of larger diameter (modal size 4mm; Fig. 3.11; $p < 0.001$) polyps throughout the ilea compared to the vehicle only treated $Apc^{min+/-}$ mice ($p < 0.001$).

The number of polyps along each segment of the intestinal tract (duodenum, jejunum, ileum, colon and rectum) was analyzed (Fig 5e). No polyps were found in any segment of the intestinal tract in *wt* mice treated with either vehicle or LSL. There were no significant changes to the number of polyps found growing along the duodenum (8 ±2 vs 9±4 $p > 0.05$ ns), colon (2±3 vs 4±4 $p > 0.05$ ns) or rectum (0±0 vs 0±0 $p > 0.05$ ns) in $Apc^{min+/-}$ mice fed vehicle or 50mg/kg LSL. There was a significant increase in the number of polyps in the jejunum (12±4 vs 21±4 $p > 0.0001$) and ileum (20±8 vs 42±9 $p > 0.0001$) in $Apc^{min+/-}$ mice fed vehicle or 50mg/kg LSL.

Histological features of sections from *wt* mouse ilea treated with vehicle or 50mg/kg LSL are characterized by evenly spaced, narrow villi with mucoid glands at their base (Fig. 3.12; top). Sections through $Apc^{min+/-}$ polyps (Fig. 3.12b; bottom) treated with vehicle-only (left) or 50mg/kg LSL (right) reveals a severely disturbed villous architecture

Figure 10: LSL induces anal polyps in $Apc^{min+/-}$ mice.

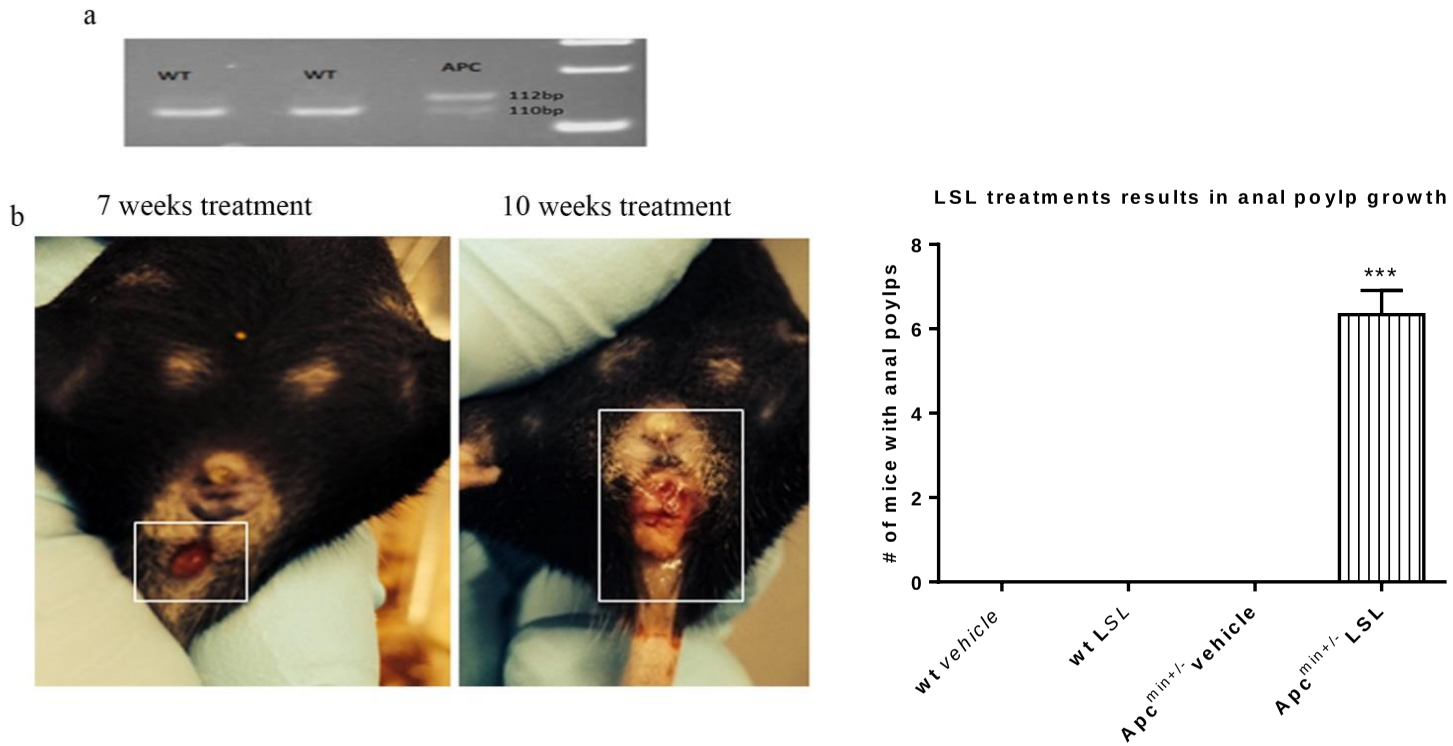
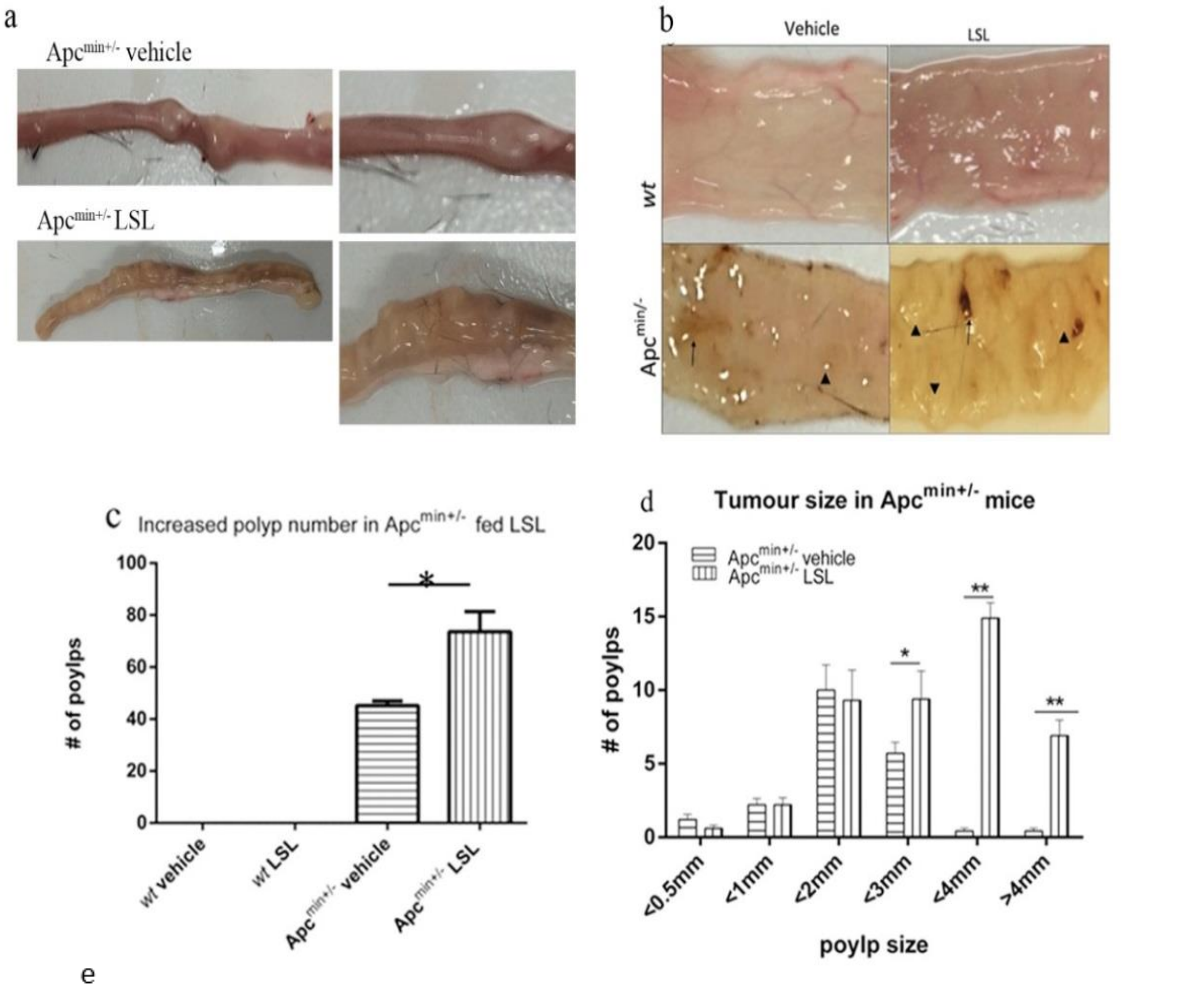


Fig 3.10: PCR and restriction enzyme analysis of genomic DNA from wt or $Apc^{min+/-}$ littermates (a); *wt* band : 111bp, heterozygous $Apc^{min+/-}$ mouse - 111 & 123bp. (b) After 7 weeks of 50mg/kg LSL treatment, bloody mucus and small polyps (0.3mm +/- 2) was observed growing from the anal cavity (n=6/10 mice). By week 10 of LSL treatments, anal polyps were 1-2mm in size and there were obvious signs of bleeding from the intestinal tract. Values represent mean ± SEM (n=10/group)

Figure 3.11: LSL increases polyp number and size in Apc$^{min+/-}$ mice



e

Quantification of polyp location				
	wt vehicle	*wt* LSL	Apc$^{min+/-}$ vehicle	Apc$^{min+/-}$ LSL
Duodenum	0 ± 0	0 ± 0	8 ± 2	9 ± 4
Jejunum	0 ± 0	0 ± 0	12 ± 4	21 ± 4 ***
Ileum	0 ± 0	0 ± 0	20 ± 8	42 ± 9 ***
Colon	0 ± 0	0 ± 0	2 ± 1	4 ± 4
Rectum	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Fig 3.11: Mice were fed either LSL or vehicle-only every other day for 70 days. a; Examination of low power images of whole intestinal segments from Apc$^{min+/-}$ mice. There are visible polyp growth along the intestinal tract. Vehicle treated Apc$^{min+/-}$ mice exhibit a healthy mucosal coating while LSL treated mice exhibit a visible yellow colouring to the mucosal membrane with an abundance of polyp growth distorting the intestinal tract shape. b; *wt* mice treated with vehicle or 50mg/kg LSL revealed no discernible differences in gross morphology (top). However, in Apc$^{min+/-}$ (bottom), those treated with vehicle-only (left) showed evidence of occult bleeding (arrow) as well as numerous polyps (c arrow heads) with a modal diameter of 2mm (d). In Apc$^{min+/-}$ mice treated with LSL, there was evidence of recent haemorrhage (arrows) as well as significant increases in both polyp number (c;*$p < 0.01$) and size (d;**$p < 0.001$) compared to vehicle-only controls. e; Segments of the intestinal tract was analysed to compare tumour number. Values represent mean ± SEM(n=10/group. significance was determined by an one-way ANOVA or students T-tests.

Figure 3.12: Histological analysis of intestinal segments from $Apc^{min+/-}$ mice

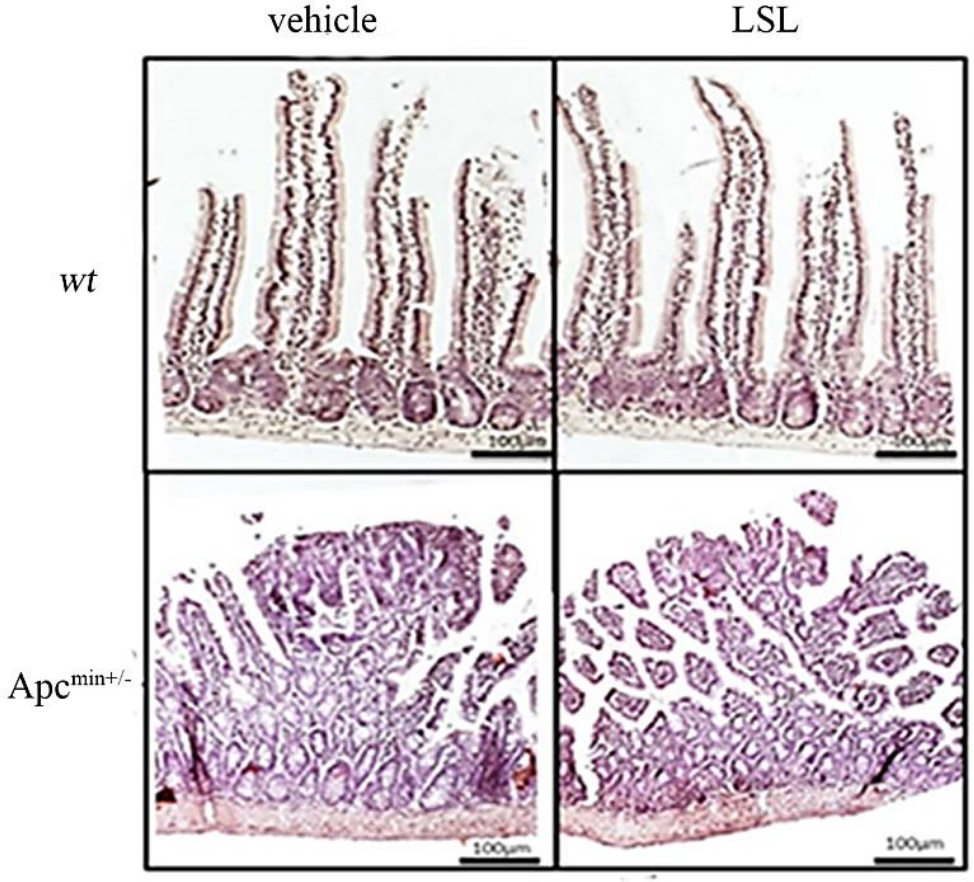


Fig 3.12: Histology sections from *wt* (top) or $Apc^{min+/-}$ (bottom) stained with Haematoxylin and Eosin. Distinct vili in *wt* treated with vehicle (left) or 50mg/kg LSL are seen. In $Apc^{min+/-}$ littermate mice, villous structures are grossly pertubed withing polyps in both vehicle-treated mice (bottom left) as well as those treated with 50mg/kg (bottom right).

LSL treatment specifically increases splenic weight and red pulp proportion and decreases Payer's patch numbers in $Apc^{min+/-}$ mice

Feeding *wt* mice with either vehicle-only or 50mg/kg LSL, also did not affect the wet weights of excised spleen (Fig. 3.13a,b; NS, $p > 0.05$). However, spleens from $Apc^{min+/-}$ mice were both larger (Fig. 3.13a) and heavier (Fig. 3.13b; $p < 0.0001$) than those from *wt* mice. Administration of 50mg/kg LSL for 70 days to $Apc^{min+/-}$ mice also resulted in a statistically significant increase in splenic size (Fig. 3.13a) and weight (Fig. 3.13b; $p < 0.05$). Examination of histological sections from *wt* mouse spleen (Fig. 3.13c; top) revealed conspicuous intensely basophilic areas of white pulp, separated by less dense regions of red pulp in the areas responsible for removal of old or damaged erythrocytes. In $Apc^{min+/-}$ mice the proportion of red pulp was increased compared to *wt* (*c.f.* Fig. 3.13c top and middle; $p < 0.05$). Following treatment with 50mg/kg LSL there was a further increase in red pulp size as compared to vehicle-only controls (*c.f.* Fig. 3.13c middle and bottom $p < 0.05$).

$Apc^{min+/-}$ mice had a lower number of Peyer's patches (PP) along the intestinal tract (Fig. 3.14). There were no significant difference between *wt* vehicle and LSL (11 vs 10; ns, $p > 0.05$). $Apc^{min+/-}$ had reduced PP numbers compared to vehicle (4.4 vs 11 **p <0.002). LSL dosing significantly decreased PP numbers compared to vehicle (1.4 vs 4.4 * p <0.01) (Fig 3.14b).

Figure 3.13 LSL treatment causes splenomegaly and an increase in red pulp area in Apc$^{min+/-}$ mice

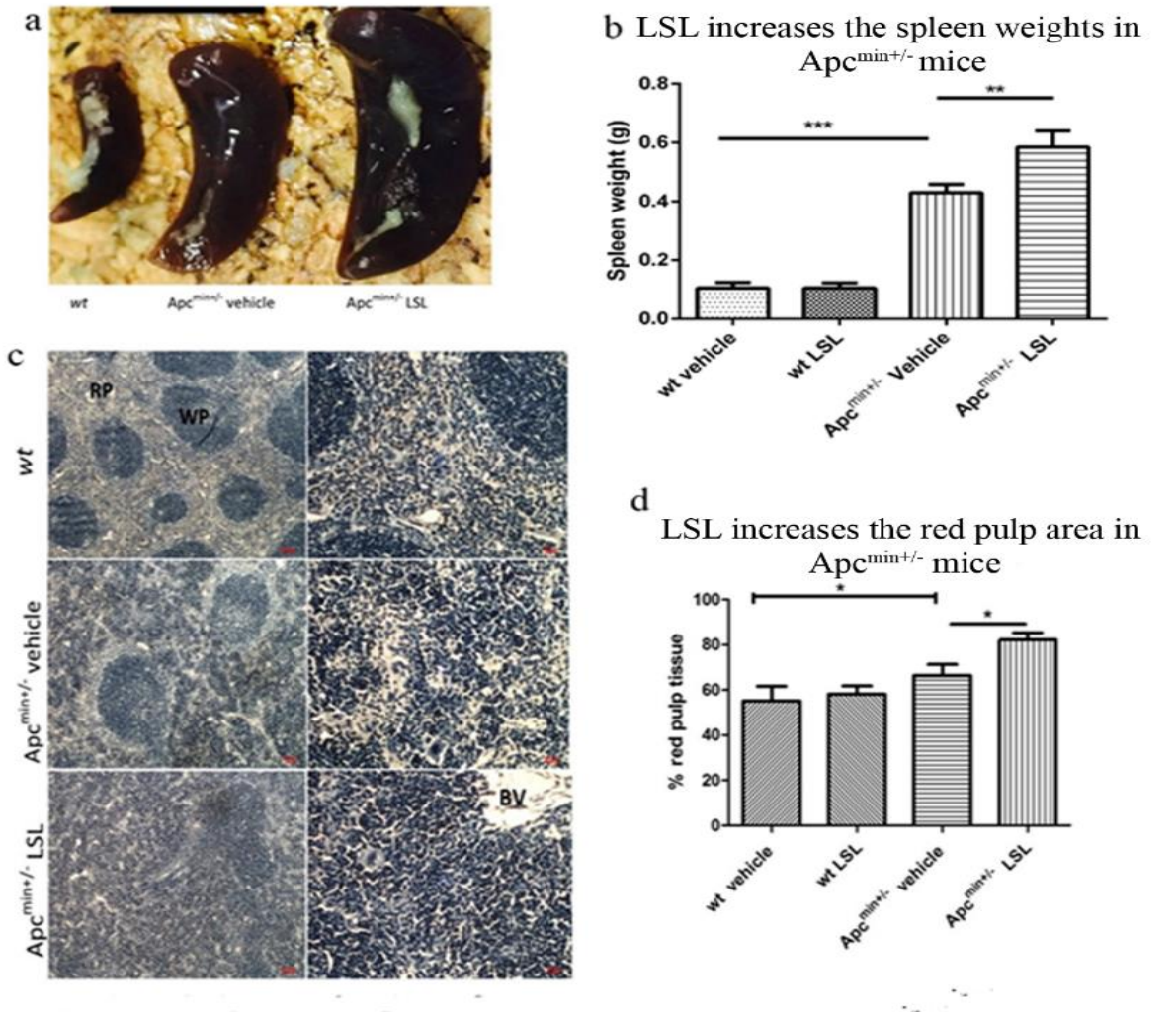


Fig 3.13 a; Photographs of dissected spleen from a *wt* mouse and Apc$^{min+/-}$ fed with vehicle or LSL. b; Apc$^{min+/-}$ mice had a significantly greater spleen weight compared to *wt* mice (**** $p<0.00001$). LSL administration resulted in a significant increase in splenic weights compared to the vehicle control mice (* $p<0.01$). Splenic histology from *wt* mice (c top), Apc$^{min+/-}$ mice fed vehicle control only (c middle) and Apc$^{min+/-}$ fed LSL (c bottom). WP = White pulp, RP= Red pulp, BV= Blood vessel. Apc$^{min+/-}$ mice demonstrated altered splenic pathology with increased red pulp regions (haematopoietic rich tissue). LSL fed mice showed a further change in histopathology (c.f c middle vs bottom) with spleens demonstrating a significant increase in red pulp (* $p<0.05$). Graphs represented of mean ± SEM. Animals per group n=10. Statistical significance was determined by students T-test.

Figure 3.14: LSL decrease Peyer Patch numbers along the intestinal tract in Apc$^{min+/-}$ mice

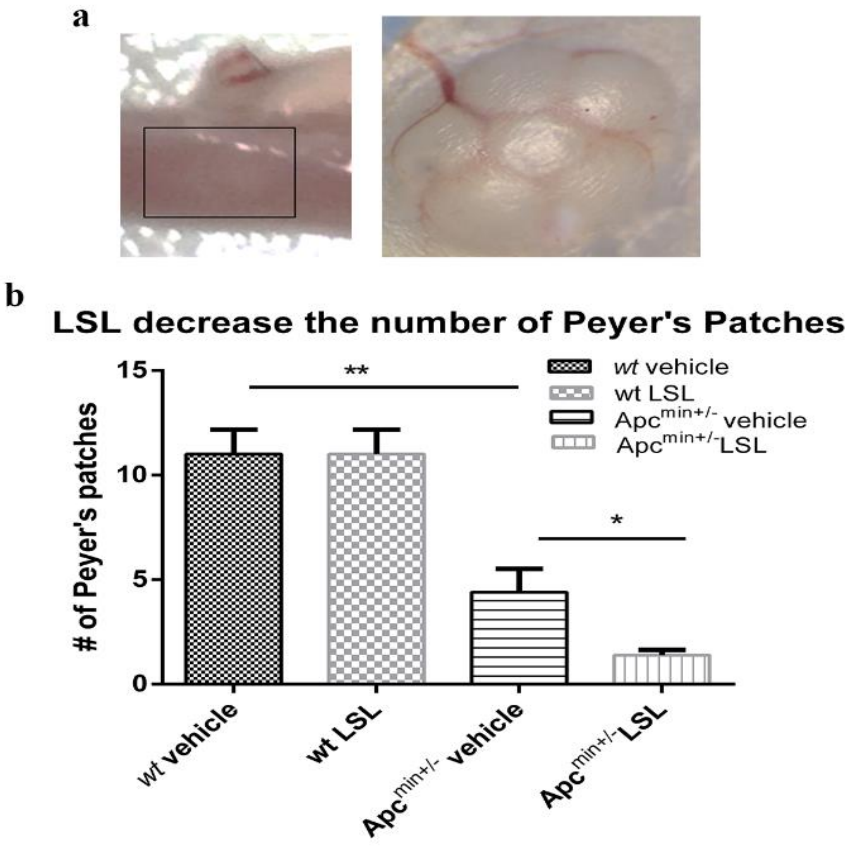


Fig 3.14: a; Photographs of the apperance of a Peyer patch (PP) found on the outside of the intestinal tract. b; Quantification of the number of PP found in *wt* or Apc$^{min+/-}$ mice treated with vehicle or LSL. There were no significant difference between *wt* vehicle and LSL (11 vs 10 ns). Apc$^{min+/-}$ had reduced PP numbers compared to vehicle (4.4 vs 11 **p <0.002). LSL dosing signifcantly decreased PP numbers compared to vehicle (1.4 vs 4.4 * p <0.01). Statistical significance was determined using students t-test. Values represent mean +/- SEM. n=10 per group.

LSL significantly decreases haematocrit levels in Apc$^{min+/-}$ mice.

The haematocrit levels of both *wt* and Apc$^{min+/-}$ mice were determined at both the mid-point of LSL dosing (day 35) (Fig 3.15a) as well as at time of euthanasia (day 70) (Fig 3.15b). At 35 days there were no significant changes in haematocrit levels between *wt* and Apc$^{min+/-}$ mice (49.0 ± 0.5 vs 50.0 ± 1.0; NS, $p > 0.05$). There were also no significant differences observed between vehicle-only or LSL treatment groups (47.5 ± 1.2 vs 48.0 ± 1.2; NS, $p > 0.05$). Haematocrit values were significantly higher in *wt* than Apc$^{min+/-}$ mice (49.5 ± 0.9 vs 38.1± 1.2; $p < 0.001$). Additionally, the feeding of Apc$^{min+/-}$ mice with 50 mg/kg LSL for 70 days caused a significant decrease in haematocrit compared to the vehicle-only control (38.1± 1.2 vs 28.2 ± 1.8; $p<0.05$).

Figure 3.15: LSL decreases the haematocrit levels in Apc$^{min+/-}$ after 70 days treatment

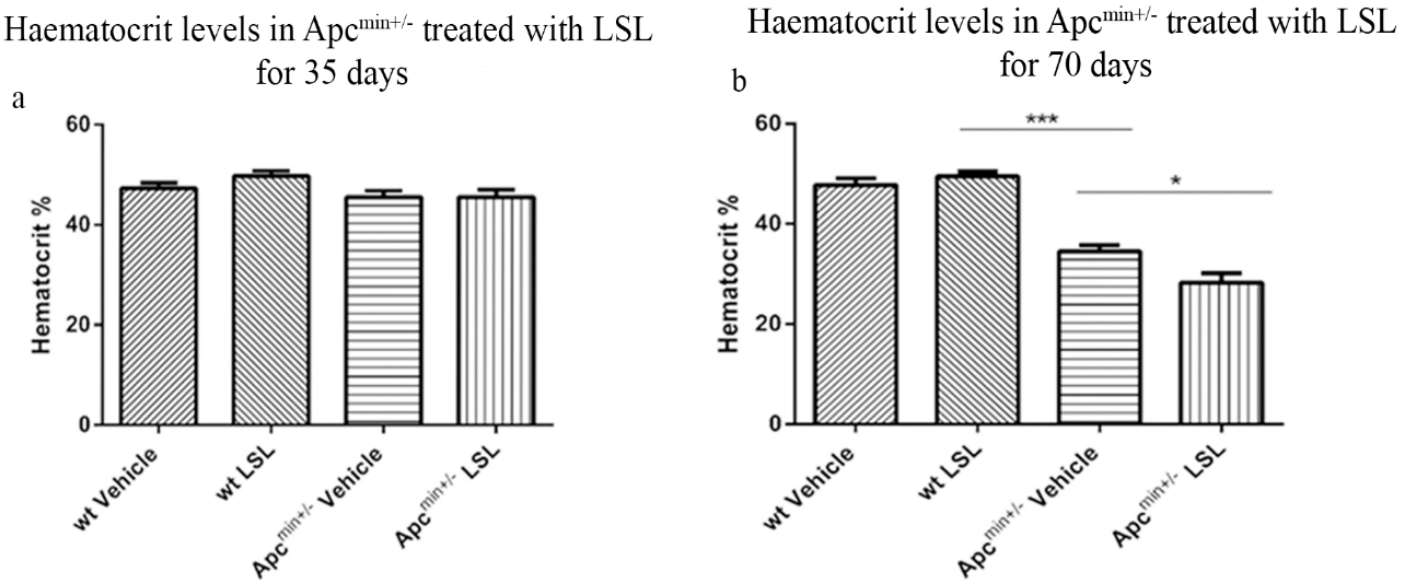


Fig 3.15 . Haematocrit measurements from *wt* or Apc$^{min=/-}$ mice fed vehicle control or LSL after 35 days (a) or 70 days (b) of treatment. After 35 days of LSL treatement there were no significant differences in haematocrit levels between *wt* or Apc$^{min=/-}$ mice and no difference between treatment groups. After 70 there was a significant reduction in haematocrit levels in Apc$^{min=/-}$ compared to *wt* mice (49.5 ± 0.9 vs 38.1± 1.2 *** p <0.0001). LSL treatments caused a furthur decline in haemocrit levels in Apc$^{min=/-}$ compared to vehicle control mice (38.1± 1.1 vs 28.2± 1.8 * p <0.01). Statistical analysis determined using students t-test. Values represented by mean ± SEM. n=10 per group.

3.5 Discussion

In order to decrease dependency on petrochemical derived surfactants, biosurfactants are increasingly finding use in a variety of applications ranging from industrial and household cleaning reagents through to skin-care products and foodstuffs (Levin and Miller, 2011). The organism with the highest productivity yield of biosurfactants is the pathogenic species *Pseudomonas aeruginosa*, which has made their large scale industrial and health-care use problematic (Lang and Wullbrandt, 1999). Modified strains of the yeast *Starmerella bombicola* are a potential commercially viable alternative, as it is non-pathogenic and a high yielding producer of homogenous SL In addition to their current commercial uses, SL preparations have previously been reported to have anti-cancer activity based on their ability to reduce the viability of pancreatic (Fu et al., 2008), lung , liver (Chen et al., 2006a) and esophageal cancer cells (Shao et al., 2012) *in-vitro*. However, these aforementioned studies are difficult to compare, as inter-study variation is significant and the purity as well as homogeneity (proportion of SL species) is often unreported. Since both purity of SL as well as homogeneity (Cavalero and Cooper, 2003) can affect the outcome of biological responses to these molecules, we produced a large batch of pure (99% SL) and homogeneous LSL preparation (96% C18:1) which was used for all subsequent use in *in vitro* and *in vivo* experiments. SL are normally formed as a complex mixture with related species differing by the degree of sophorose acetylation as well as fatty acid length and saturation (Otzen, 2016). This species diversity, coupled with the various congeners found in crude SL preparations makes separation and purification difficult, demanding and expensive; however, it is vital when considering potential pharma-therapeutic uses (Rodrigues et al., 2006). In the past number of years, purification of SL species have been achieved by the use of thin layer

chromatography, HPLC and column chromatography (Develter and Lauryssen, 2010). The majority of studies investigating the anti-cancer potential of SL separate and purify samples with the use of HPLC, MS or NMR (Fu et al., 2008, Chen et al., 2006b) producing data showing the exact composition of the SL to be tested, but the information on purity and composition is often omitted. One notable study (Ribeiro et al., 2015) which used pure and well characterized SL (92% 18:1 LSL) examined their effects on breast carcinoma cells and found a dose-dependent cytotoxic effect. Here we report on a 96% 18:1 LSL preparation that was used throughout both our *in vitro* and *in vivo* experiments.

We addressed whether a pure preparation of LSL from *Starmerella bombicola* has a differential and/or dose-dependent effect on transformed adherent cells in comparison to "normal" adherent cells; a highly desirable property for potential cancer chemotherapeutics. We assessed five well characterized colorectal cancer cell lines (HT29, HT115, HCT115, LS180 and CaCo2) in addition to adherent, non-transformed colonic epithelium (CCD-841-CoN) and lung fibroblasts (MRC5). The 96% 18:1 LSL preparation used in our experiments had a differential effect on induction of cell death in these cell types; more specifically, they have a more potent effect against "normal" cells at lower doses (10µg/ml). Only a small number of studies have been carried out looking at the anti-cancer activities of SL isolated from *Starmerella bombicola,* such as that described in the breast cancer line MDA-MB-231 (92% C18:1 LSL). The majority of studies have been carried out using SL produced by *Wickerhamiella domercqiae*, which was recently identified as *C. bombicola* after genome sequencing (Li et al., 2016b). These cytotoxicity studies (Chen et al., 2006a), also demonstrate a similar potent effect of SL from doses ranging from 40µg/ml – 2mg/ml (compared our study: 3—60µg/ml). The wide range of dose efficacy may be partially

explained by the differences in SL species and uncharacterized mixtures. The repeatability and high level of consistency in the data from our *in vitro* and *in vivo* studies is consistent with our conclusions on the biological activity of our LSL sample, although we cannot exclude the possibility that the 3.8% ASL found within our SL mixture may have some co-incident biological activity (see Chapter 4).

In one previous study, LSL mixtures demonstrated no effect on circulating (non-adherent) blood monocytes, although their comparison with adherent pancreatic tumour cells is spurious (Fu et al., 2008). Other non-transformed cell lines examined in the literature include the uncharacterized, and not readily available HL7702 and the 'Chang' liver cells, believed to derived from normal liver, but later found to be HeLa contaminated (Masters, 2002).

We determined that colorectal cells treated with 40 – 70µg/ml LSL begin to die after 24hr *in vitro*. The predominant type of cell death observed, following ethidium bromide/acridine orange staining, was morphologically consistent with necrosis. This morphological necrosis occurred at doses of 70µg/ml in the cancer cell lines and 20µg/ml in the normal cell lines. Necrosis is a type of unregulated programmed cell death (Ouyang et al., 2012), characterized by the disruption of the lipid membrane resulting in the leakage of intracellular proteins, reduction in ATP and cell lysis thus provoking an immune response (Kumar et al., 2012). SL induced necrosis has been demonstrated in other cell lines, as quantified by LDH release: such as HPAC (Fu et al., 2008) and the HL-60 leukemic cell line (Isoda et al., 1997). The induction of necrosis in various cell lines (including those described in this study) likely occurs *via* the intercalation of BS into the lipid bilayer as

previously documented (Isoda et al., 1997). Koley *et al*., (2010) explained that, at a cell-line specific minimal concentration, surfactants integrate into the cell lipid membrane, resulting in carbon chain structural rearrangement. High doses of SL induce tension at the interfacial region of the bilayer, resulting in phospholipid dehydration which affects lipid stability, cellular adhesion and function (Koley and Bard, 2010, Fracchia et al., 2012). This disruption of the lipid bilayer, has also been described as the mechanism of action from SL-induced cell death in human sperm (Shah et al., 2005).

We also tested the migration inhibitory potential of LSL on CRC cell lines *in vitro.* Doses of 10 and 50µgml were chosen based on the previous cell viability and cell death assay as a concentration which could have a potent effect without resulting in a change in viability or morphology across the cell lines. At both 10 and 50µg/ml, there was a significant decrease in migratory activity of CCD-841-CoN cells. However, in HT29 and HT115 cells, a reduction in migration only occurred at the higher 50µg/ml dose. Studies carried out by Ribeiro and co-workers have shown that LSL decrease migration in the scratch assay, a measure of migration, in breast cancer MDA-MB-231 cells at a dose of 5mg (Ribeiro et al., 2015).

Cancer cells are renowned for possessing the ability to invade through an extracellular matrix; a key hallmark for the progression to metastasis (Hanahan and Weinberg, 2011). Therefore, we tested the ability of LSL to inhibit invasion through Matrigel (Nicosia and Ottinetti, 1990) of HT29 and HT115 cell lines. Similar to the results from our migration study, only the higher 50µg/ml concentration of LSL resulted in a decrease in the percentage of cells that invaded through the Matrigel. LSL demonstrated more effective

inhibition of invasion against HT29 cells compared to HT115, however this may be attributable to the metastatic carcinoma phenotype associated with the HT115 cell line (Jonas et al., 1996) (Parr et al., 2000).

The *in vitro* data presented in this chapter clearly demonstrates the ability of LSL to reduce the viability of normal and cancer cells and disrupt the migration and invasion of cancer cells at high concentrations. However, this study would have benefited from the addition of extra intraexperimental controls. This includes the addition of a well characterised chemotherapeutic such as etoposide or doxorubicin to compare the efficacy of LSL in reducing cell viability and seeing how that compares to the gold standard chemotherapeutic drugs. It would be suggested that this would be included in future repeats of the experiments.

Studies investigating the therapeutic potential of SL in *in vivo* models are limited, with the exception of models describing their potential for reduction of sepsis. SL mixtures reduce mortality in rats with experimentally induced sepsis induced *via* caecal puncture. However, in comparison to the crude SL mixtures, LSL caused an unexpected increase in the mortality rate in rats with septicaemia at the same dose (5mg/kg) (Hardin et al., 2007, Bluth et al., 2006).

The $Apc^{min+/-}$ mouse is a widely used animal model for investigating the correlations between food, genetics and chemotherapeutic intervention in the development of intestinal adenomatous neoplasms (polyps). These mice have a life span of <150 days due to secondary consequences of the disease (extensive bleeding of colonic polyps accompanied by anemia), this is one of the reasons it makes an ideal and quick model to study the effects

of compounds (Yekkala and Baudino, 2007). Oral administration was chosen as the ideal route of administration, in contrast to a traumatic abdominal injury, as it allows the LSL to have direct access to the gut epithelium and polyps to exert their biological effect.

SL have been reported to be well tolerated and non-toxic in mice at doses below 15g/kg (Ikeda et al., 1986). To confirm this prior to embarking on a full-scale study, we tested if the oral administration of 0.5, 5 and 50mg/kg LSL would be well tolerated by *wt* (C57Bl/6j) and $Apc^{min^{+/-}}$ mice. The behavior, weight development, food and water intake were not significantly different between treatment groups and there was no difference in the weights or gross morphology of the major organs after 5 weeks of treatments. On the basis of this pilot study we treated, we treated *wt* C57 and $Apc^{min+/-}$ mice with 50mg/kg of LSL for a total of 70 days.

Our pilot data, in addition to the long-term administration study of LSL clearly demonstrates that oral administration has no detrimental effect on behavior, weight, or food/water consumption at doses of 0.5, 5 and 50mg/kg. Considering the ability of SL mixtures to reduce cancer cell viability, it is surprising that there is only one other *in vivo* study investigating the use of SL in a pre-clinical model of cancer (Li et al., 2016a). In the aforementioned study, BALB/c nude mice were subcutaneously injected with HeLa cells and either 5, 50 or 500mg/kg of SL mixtures were administered by intragastric gavage for 12 days. Tumour analysis revealed that treatments with either 5, 50 or 500mg/kg SL inhibited tumour growth by 29%, 41% and 52% respectively (Li et al., 2016a).

Our studies indicated that LSL has a potent effect on survival, migration and invasion of on CRC and normal cell lines at a range of concentrations starting from 20µg/ml and was well

tolerated via oral administration *in vivo*. We, therefore hypothesized that long-term treatments (70 days) would slow the progression of colorectal polyps in the $Apc^{min+/-}$ mouse model. Our results conclusively show that orally administered purified LSL do not decrease polyp development in this model. In contrast, LSL causes an exacerbation of growth of adenomatous polyps in the intestinal pre-cancerous $Apc^{min+/-}$ mouse model. After 7 weeks of treatment, small polyps were also visible growing from the anus and by 10 weeks, the anal polyps had a 3-fold increase in size and there were obvious signs of bleeding.

The number of polyps along each segment of the intestinal tract was individually counted and compared. Interestingly the number of polyps quantified in each segment of the vehicle treated $Apc^{min+/-}$ mice were in line with other published studies (Song *et al*., 2000). It was noted that LSL administration resulted in a significant increase in the number of polyps found in the jejunum and ileum of LSL treated $Apc^{min+/-}$ mice. The biggest difference between the different segments of the small intestine is the pH levels. As it moves away from the gastric acid levels in the stomach the pH begins to rise. The duodenum has a pH of 4-6, while the jejunum and ileum has a pH 6-7. LSL has a neutral to basic pH by nature, therefore it could be hypothesized that an acidic environment can alter the biological activity of LSL. This could be tested further in the future by changing the pH of the LSL solution and testing its biological activity on cells using an MTT assay or running it through an HLPC-MS to look for any changes,

LSL treatment also increased the size (volume) of intestinal polyps, a method that is currently used as an indicator of polyp burden in the $Apc^{min+/-}$ mouse model (Goodlad et al., 2006). The severity of the polyp burden is readily apparent by visual inspection of the

unopened intestinal tract of $Apc^{min+/-}$ mice fed LSL compared to the vehicle control (see Fig 3.10a), with the large numbers and increased sizes of polyps resulting in the disfiguration and expansion of the intestinal lining. It is highly likely, that continuation of the feeding of LSL in this experiment, would have hindered their ability to consume and process food, further reducing the life span of these mice (Weindruch et al., 1986).

Other markers of disease progression in $Apc^{min+/-}$ mice have been reported, with an enlarged spleen and reduced hematocrit as a result of colorectal bleeding (Yekkala and Baudino, 2007, You et al., 2006). Our study showed that LSL administration resulted in a significant increase in splenic size. Histological analysis also revealed that LSL treatment resulted in a further increase in red pulp regions in the $Apc^{min+/-}$ mice and a reduction in the number of Peyer's patches present in the intestinal tract lining. The reduction in hematocrit levels is a late stage indicator of disease progression (Moser et al., 1995). as there was change in levels following 35 days of LSL dosing in this experiment. The reduced hematocrit, a sign of anemia may explain the increase in spleen size due to the role it has in clearing out dead and defective erythrocytes (Crosby, 1959; Cesta, 2006). The increasing splenic size has also been documented by Petrioianu et al., when benzo(a)pyrene, an immunomodulatory drug was administered in mice (Petroianu, 2007). It is believed that this may be a consequence of increased lymphatic damage associated with a decrease in Peyer's Patch numbers along the intestinal tract (You et al., 2006).

In conclusion, LSL induce cell death in 5 CRC cell lines in addition to 2 normal cell lines and while oral dosing is well tolerated, our studies indicate that a dose of 50mg/kg increases polyp progression and reduces hematocrit in the pre-clinical $Apc^{min+/-}$ mouse model. This chapter would benefit from the addition of a control agent to compare the effects of the LSL

with. As polyps that grow along the intestinal tract in FAP patients are benign – they are often not treated with chemotherapeutics unless they become malignant. Currently in the clinic Celebrex, a cyclooxygenase-2 inhibitor, has been approved to help decrease polyp burden in patients that are in the high-risk category for developing CRC (Steinbach *et al*., 2000). It has also been shown to reduce the polyp burden significantly in the $Apc^{min+/-}$ mouse model (Jacoby *et al.*, 2000). Therefore, in future studies, Celebrex could be used as a control to compare against the efficacy of SL.

This study would also benefit from extra experiments in the future. This chapter would benefit from a time course study. This chapter looks only at the effects on cell viability after LSL application for 24hrs. Future studies could examine if the SL still has an effect after 48 and 72hrs. It would also be interesting to see how quick SL affected cell viability and induced cell death. This could be carried out by stopping the experiment in increasing increments until viability is altered. It would be first carried out on a large scale using a MTT and stopping the assay every 15 minutes which would give an approximate time scale. This could then be applied to the cell death assay.

Chapter 4

Purified acidic sophorolipids selectively inhibit colorectal tumoucell growth *in vitro* and restore haematocrit levels in $Apc^{min+/-}$ mice

4.1 Introduction

Compounds that are non-toxic, orally tolerated and specifically target epithelial neoplastic cells (Kelloff et al., 1994) in the intestinal tract could have great potential in delaying progression of intestinal neoplasms; in particular, those that are associated with progression to colorectal cancer (D'Incalci et al., 2005) such as Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC)/Lynch syndrome. Currently, the gold standard for treatment of FAP/HNPCC is surgery, followed by adjuvant chemotherapy (O'Connell et al., 2010). Adjuvant chemotherapy does not discriminate between normal and transformed tissue, which leads to a variety of potentially serious complications including cardiotoxicity, immune dilapidation and neurotoxicity (Morgan and Rubin, 1998, Mazevet et al., 2013).

The $Apc^{min+/-}$ mouse is a widely used model of FAP as it recapitulates key pathological features of the human disease (Hinoi et al., 2007) and provides a useful tool to investigate the effects of genetics, diet and therapeutic drugs on tumorigenesis in the gastrointestinal tract. Similar to human FAP, it was first noted that $Apc^{min+/-}$ mice develop adult-onset anaemia with haematocrit levels <45%, the passage of bloody stools and have a reduced life span, with post mortem analysis revealing the growth of numerous tumours along the small intestine (Moser et al., 1995). The $Apc^{min+/-}$ mouse model has also been widely used to test the effects of chemotherapeutics on tumour growth and development such as NSAIDs (Aspirin) (Jacoby et al., 2000). In addition, the $Apc^{min+/-}$ mouse model has also be used to develop a further understanding of clinically used chemotherapeutic drugs such as 5-fluorouracil (Tucker et al., 2002), which is widely used in the treatment of CRC. The cytotoxity of chemotherapeutic drugs such as 5-fluorouracil are similar in mouse models as well as human trials; the side effects including weight-loss, hair-loss,

myelosuppression and cardiovascular toxicity (Steger et al., 2012, Popovic et al., 1981). Although this specific range of side effects are unique to 5-fluorouracil, off target effects of clinically used chemotherapeutic agents, such as doxorubicin (Avilés et al., 1993) , bleomycin (Cohen et al., 1973) and cyclophosphamide are well recognised. It has been suggested that use of naturally derived compounds as alternatives to synthetic drugs may help reduce this bystander tissue toxicity. A number of naturally derived compounds are already in widespread use in cancer treatment such as Taxol, first derived from the bark of the yew tree (Stierle et al., 1993), although demand for this single species chemotherapeutic agent has necessitated synthetic production in the laboratory (Flam, 1994). This has also has been seen in other drugs such as vinblastine, morphine and digoxin, to name a few (Fabricant et al.,2001). It has been hypothesised that drugs dervived from naturally occurring sources and contain highly purified levels of the naturally occurring biologically active agent may reduce toxicity and side effects (Beutler, 2009) i.e. 94% ASL, as used in this chapter.

Similar to the crude preparations of natural compounds (such as Yew bark described above), naturally occurring SL mixtures contain a variety of amphiphilic species, composed of a hydrophobic fatty acid (C16- C18) tail and a hydrophilic carbohydrate sophorose head, and made up mostly of ASL or LSL. The anti-cancer properties of SL have received a lot of attention in recent years, as they show cytotoxic effects in human pancreatic (HPAC) (Fu et al., 2008), liver (H7402), lung (A549) (Chen et al., 2006a), brain (LN229,HNCG-2) (Dhar et al., 2011), esophageal (KYSE109, KYSE450) (Shao et al., 2012), breast (Ribeiro et al., 2015), cervivcal (HeLa) (Li et al., 2016) and leukemic (HL60,K562) (Chen et al., 2006b) cell lines *in vitro* and a potential tumour shrinking capability in an *in vivo* model of cervical cancer (Li et al., 2016). We have previously shown that a highly purified preparation of LSL results in the exacerbation of tumour

growth in the intestinal tract of $Apc^{min+/-}$ mice and secondary consequences including splenomegaly and reduced haematocrit levels (Callaghan et al., 2016).

To date, very few *in vivo* bioactivity studies have been reported for SL mixtures, although toxicology experiments have shown SL is well tolerated in mice, non-irritant when topically applied to the skin and eyes of rabbits and non-toxic when administered orally to either mice (Callaghan et al., 2016) or rats (Ikea et al., 1986). SL mixtures have also been observed to reduce inflammation and increase life-span in a model of severe abdominal sepsis in rats (Hardin et al., 2007) as well as decreasing IgE levels in a murine asthma model (Bluth et al., 2008). Although SL shows promising anti-cancer activity *in vitro*, the gross composition and percentage of congeners within the SL mixture used in studies are not disclosed in a majority of investigations, although claims of LSL enriched preparations are common due to the preponderance of LSL in comparison to ASL's within naturally occurring SL mixtures (Marchant and Banat, 2012, Van Bogaert et al., 2011).

The process of synthesis produces complex molecular mixtures of SL, differing in their quantities, acetylation and saturation levels, which prove tedious to separate and purify (Asmer et al., 1988). In comparison to other SL congeners, ASL have reduced production costs as they require less yeast in the culture medium (20g/l for ASL vs 50g/l for LSL) (Casas and García-Ochoa, 1999) and are the first congener to be produced in the bioreactor when there are less favourable conditions present (i.e. less energy required for production) (Ducreux et al., 1997). The proportion of ASL within enriched preparations can be improved when the media is supplemented with polyunsaturated fatty acids and this can be further increased via simple hydrolysis of the SL mixtures which increases the number of open ring structures (Van Bogaert et al., 2007).

ASL have enhanced solubility when compared to other SL congeners (Baccile et al., 2017), which may prove useful for their use as pharmaceuticals, as they can be dissolved

in saline instead of potentially toxic solvents such as DMSO. Both solubility as well as enhanced foaming attributes of ASL contribute to their preferred use in the food, cosmetic and the bioremediation industries (Lang et al., 2000).

In order to clarify whether ASL has colon tumour cell-specific cytotoxicity, we used a well characterized homogeneous a 94% ASL composed of mostly of C18:1 and compared its anti-cancer activity with that of LSL (see Chapter 3) both *in vitro* and *in-vivo*.

4.2 Hypothesis:

A highly purified and well characterised preparation of ASL (94% pure diacetylated) selectively inhibits colorectal tumour growth *in vitro* and delays disease progression in the $Apc^{min+/-}$ mouse model.

Aims and objectives:

1) To determine the proportion and composition of congeners in a purified ASL stock produced by *Starmerella bombicola* for use in *in vitro* and *in vivo* experiments.

2) To assess the cytotoxic properties of the ASL sample on four unrelated colorectal cancer cell lines, which mimic the different stages of cancer in the clinic, in addition to two representative normal lines: normal human colonic epithelium CCD-841 CoN and lung fibroblast MRC5 *in vitro.*

3) Determine the ability of orally administered ASL to inhibit tumour growth in $Apc^{min+/-}$ mice, a well-established model of colorectal neoplasia.

4.4 Results:

Production and purification of ASL

A purified form of diacetylated ASL was produced by our collaborators for use in the *in vitro* and *in vivo* studies described in chapter 2.1 of the materials and methods section. In brief, a crude SL precursor was purchased as a typical LSL/ASL (~ 80/20) mixture (Sopholiance S, Batch N°11103A) and the ASL (Fig 4.1) were purified from this mixture as described previously (Baccile et al., 2013).

HPLC-ELSD data confirmed that the sample is composed of 94% diacetylated ASL. Figure 4.2 shows the exact composition of the ASL batch and the percentage of each congener present. On the basis of the HPLC-ELSD integrated area values: the most abundant compound (75.0%) is composed, as expected, by the acidic C18:1s. The terminal C18:1t and C18:2t congeners represent 10% each, while all other congeners are limited to less than 2%. This batch sample was stored at room temperature and solubilized in PBS (*in-vitro*) or 10% sucrose (*in-vivo*) for use in all the experiments described in this chapter.

Figure 4.1: ASL structure

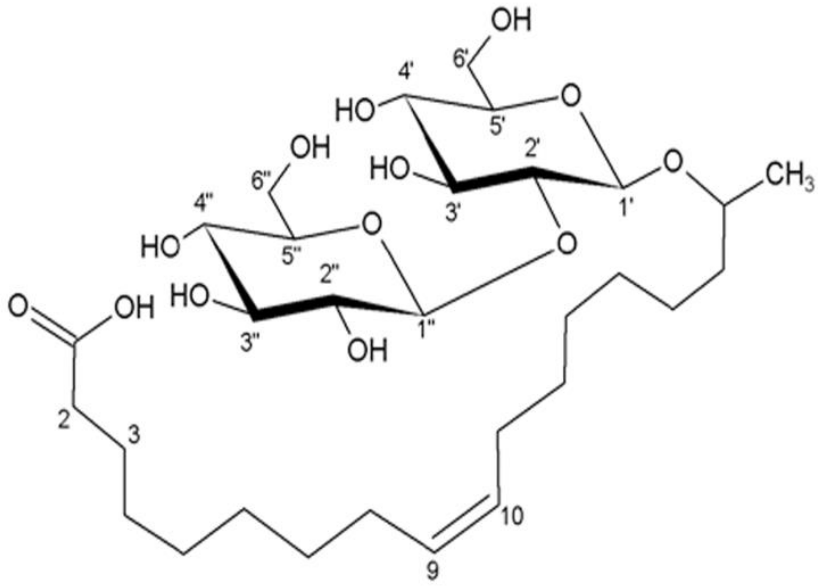
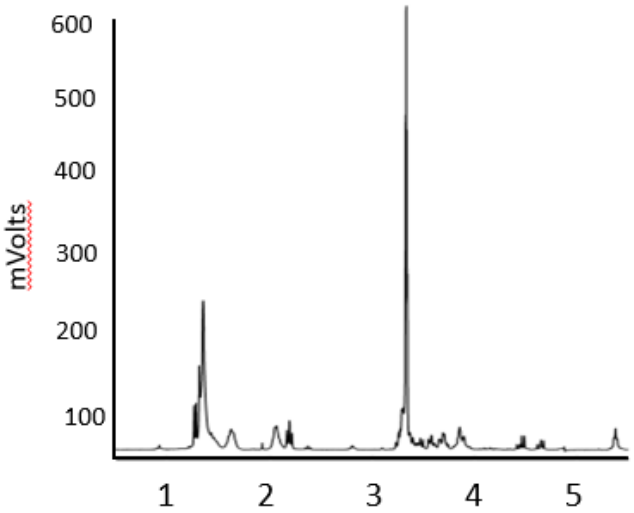


Fig 4.1: The structure of diacetylated C18:1 ASL.

Figure 4.2: The purification of ASL



Retention time	SL species, acidic non acetylated	MW	Amount in %
12.02	C16:1s	593	1.0
12.16	C16:0s	595	2.0
12.37	C16:0t	595	0.0
12.40	C18:2s	619	1.0
12.54	C18:2t	619	10.0
12.87	C18:1s	622	75.0
13.27	C18:1t	622	10.0
13.68	C18:0s	624	1.0

Fig 4.2: a) HPLC-ELSD analysis of ASL. b) The percentage of each congener present on the basis of the HPLC-ELSD integrated area values. The most abundant compound (75.0%) is composed by the acidic C18:1. The terminal C18:1t and C18:2t congeners represent 10% each, while all other congeners are limited to less than 2%.

ASL has a selective effect on colorectal cancer cell viability.

At concentrations ranging from 0.01-100µg/ml ASL did not affect the viability of either non-transformed colonic epithelial (CCD-841-CoN) or lung fibroblast (MRC5) cell lines (Fig. 4.3a) after 24h in comparison to vehicle-only control treated cultures. However, ASL concentrations above 20µg/ml resulted in a significantly reduced viability of both HT29 and LS180 cell lines ($p < 0.001$); whereas at concentrations over 40µg/ml a significant reduction in cell viability of HT115, Caco2 and HCT116 tumour cell lines was noted ($p < 0.001$).

Figure 4.3: ASL selectively decreases the viabilty of CRC cells *in vitro.*

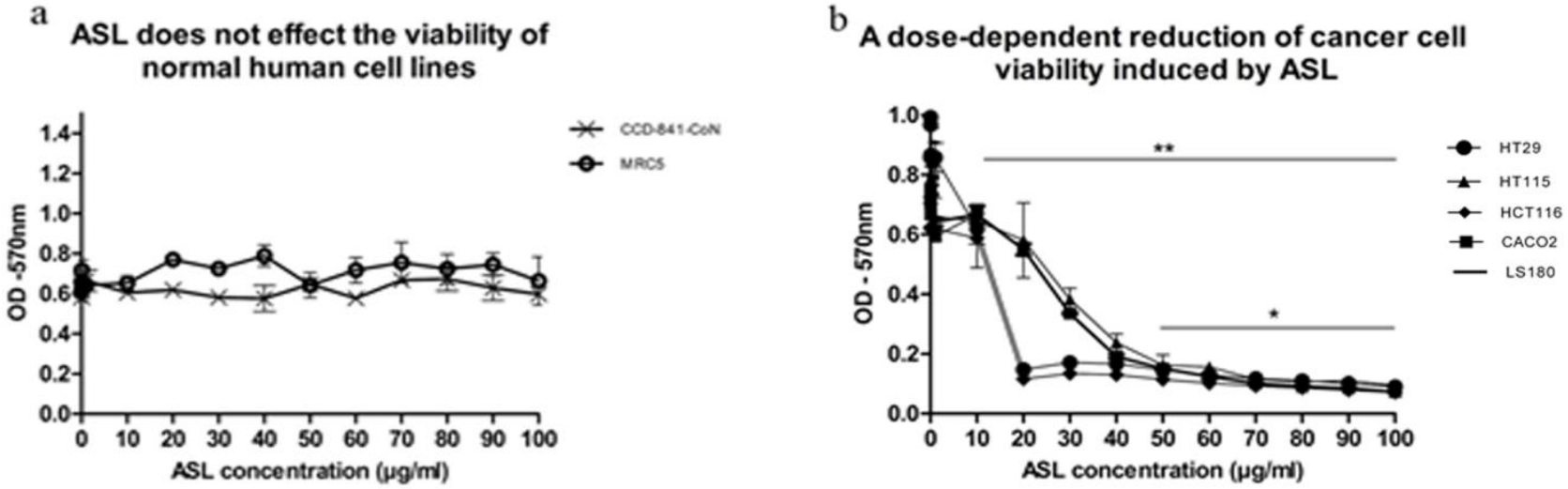


Fig 4.3: a) Following 24h of ASL treatment, there were no significant reduction in O.D 570 values at doses between 0.01-100µg/ml in control cell lines CCD-841-CoN and MRC5.b) In CRC cell lines HT29 and CACO2, there was a significant reduction in O.D 570 values from 20µg/ml ($p < 0.001$). ASL doses over 40µg/ml reduced the O.D 570 values of the remaining HT115, HCT116 and LS180 CRC cell lines ($p < 0.05$). Graphs are representative from three independent experiment repeats. Values indicate mean ± SEM (n=6). Statistical significance was assessed using an one-way ANOVA. (* $p < 0.05$, ** $p < 0.001$).

ASL induces cell rounding in CRC cells

Vehicle treated CCD-841-CoN cells grow as a confluent monolayer with a bipolar morphology following treatment with 40 or 70µg/ml ASL (Fig. 4.4), with no significant morphological features of cells in the monolayer noted being related to dose.

The CRC cell lines HT29, HT115, HCT116 and Caco2 that were treated with vehicle-only control solution display a densely packed, cobblestone-like monolayer morphology (Fig 4.4). At a dose of 40µg/ml ASL , disruption in the monolayer and detachment of CRC cells was observed. Partially detached and rounded HT29, HT115 and Caco2 cells were conspicuous within the cultures treated with 40µg/ml ASL. The HT29, HT115, HCT116 and Caco2 cells exposed to 70µg/ml ASL, demonstrated extensive monolayer disruption with distinctive cell free areas within the cultures (Fig 4.4).

To determine if the detachment of cells observed in tumour cell cultures treated with 0, 50 and 100µg/ml ASL resulted in cell death, cells were isolated from the supernatant of wells, stained with syto9 and propidium iodide and the numbers of live/dead cells counted (Fig 4.5). In the CCD-841-CoN cultures following treatment with 50 and 100µg/ml ASL there were small numbers of detached cells observed (NS; $p < 0.1$). In contrast, at 50µg/ml, there were significant increases in the numbers of dead, detached HT29 (Fig 4.5b $p < 0.0001$), HT115, HCT116 and Caco2 cells (Fig 4.5c-e respectively; $p < 0.001$). While at a dose of 100µg/ml ASL, 100% of all counted CRC cells in the supernatant were dead (Fig. 4.5b-e; $p < 0.0001$).

Figure 4.4: ASL results in morphological changes in CRC cells *in vitro*

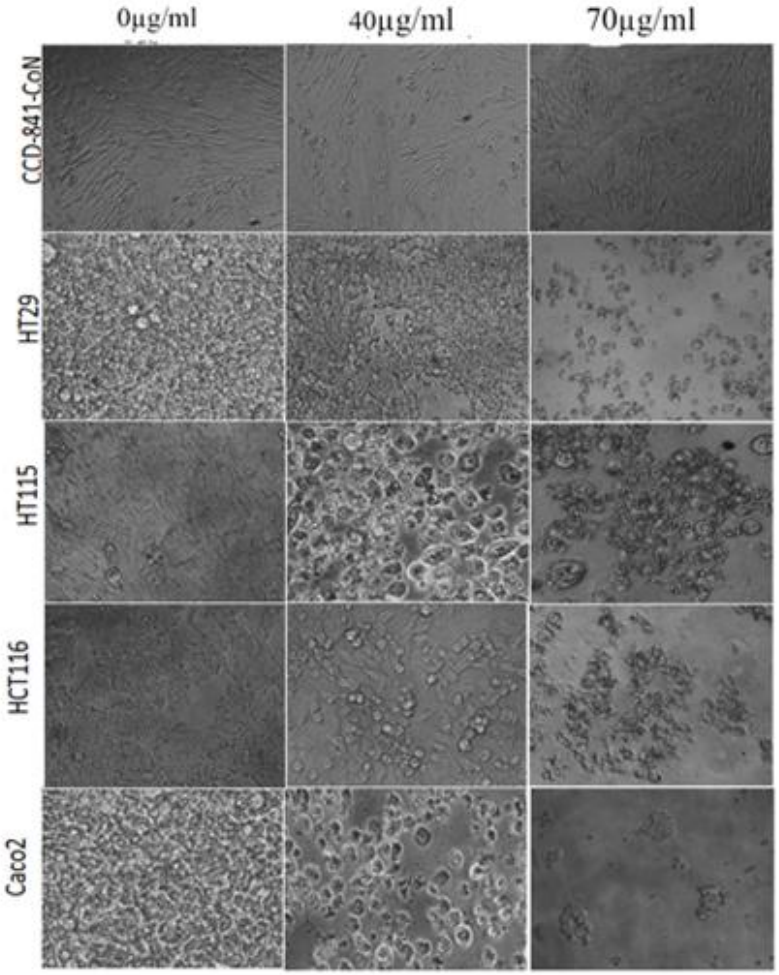


Fig 4.4: Light micrographs of normal colonc epithelial (CCD-8-41CoN) and CRC HT29, HT115, HCT116 and Caco2 cell lines treated with vehicle control or 40 or 70µg/ml ASL. 40 or 70µg/ml treatments did not induce significant changes to CCD-841-CoN cell morphology. After 40µg/ml ASL, there was obvious disruption to the monolayer with areas devoid of cells . There were also signs of cell rounding in HT29, HT115 and Caco2 cells. At 70µg/ml ASL, there was a significant increase in cell detachment in all CRC cell lines. There were obvious signs of cell rounding and fragmentation observed. Scale bar = 50µm for each image.

Figure 4.5: ASL treatments results in the detachment of dead cells

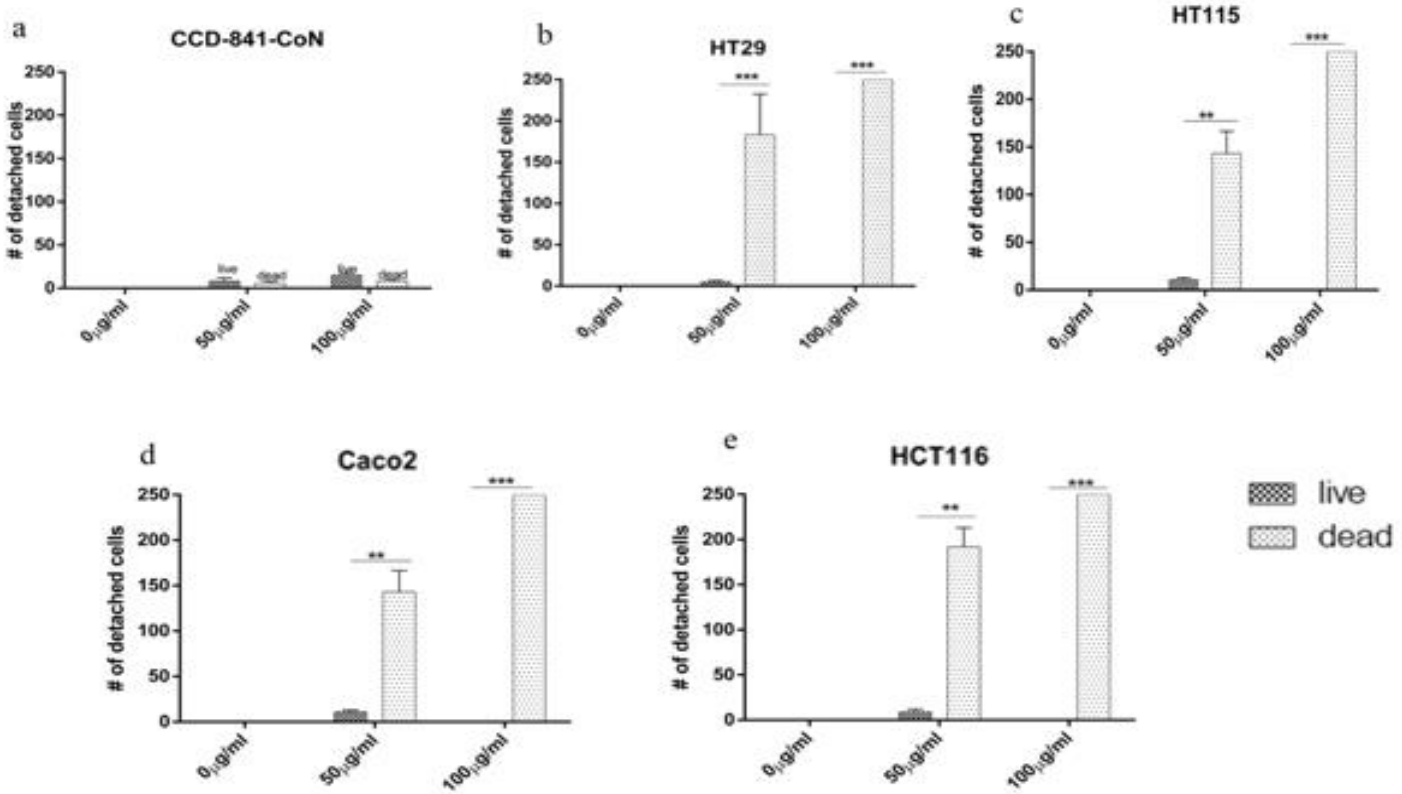


Fig 4.5: Quantification of live or dead cells using propidium iodide and Syto 9 staining on cells extracted from the supernatant of cultures treated with 0, 50 or 100µg/ml of ASL. Following treatment with 50µg/ml ASL, (a) there was a small number of deatched CCD-84l CoN cells. At 50µg/ml, 5% of HT29, 11% of HT115, 12% of HCT116 and 5% of Caco2 cells were alive. All reaining detached HT29 ($p < 0.0001$), HT115 ($p < 0.001$), HCT116 ($p < 0.001$) and Cac02 ($p < 0.001$) cells in the supernatant were dead. At 100µg/ml all detached HT29 ($p < 0.0001$), HT115 ($p < 0.0001$), HCT116 ($p < 0.0001$) and Caco2 ($p < 0.0001$) cells counted in the supernatant were dead. Graphs representative of mean ± SEM. Significance was calculated using a student's t-test.

ASL induces cell death *in vitro*.

Treatment of CCD-841-CoN cells with 20µg/ml of ASL did not result in a significant increase in the numbers of either apoptotic or necrotic cells (Fig. 4.6p; NS, $p > 0.05$); however a dose of 70µg/ml resulted in ~10% cell death within the cultures that was identified morphologically as apoptosis ($p < 0.05$).

We observed a dose-dependent increase (starting with doses as low as 20µg/ml) in cell death in all four human colorectal cancer cell lines following ASL treatment (4.6q-t). At a dose of 20µg/ml, there were equivalent numbers of apoptotic and necrotic cells in HT29, HT115 and HCT116; however, there was a higher number of apoptotic cells in Caco2 cells ($p < 0.01$).

Exposure of cultures to 70µg/ml ASL, resulted in a markedly reduced number of adherent CRC cells available for quantification (Fig. 4.6l-o). HT29 and HT115 cultures showed a significant increase in the numbers of apoptotic and necrotic cells ($p < 0.001$, $p < 0.01$ respectively) while HCT116 cells were predominantly necrotic ($p <0.0001$) and Caco2 cells had a higher number of apoptotic cells ($p <0.001$) compared to vehicle control only.

Figure 4.6: ASL induced both necrosis and apoptosis in vitro.

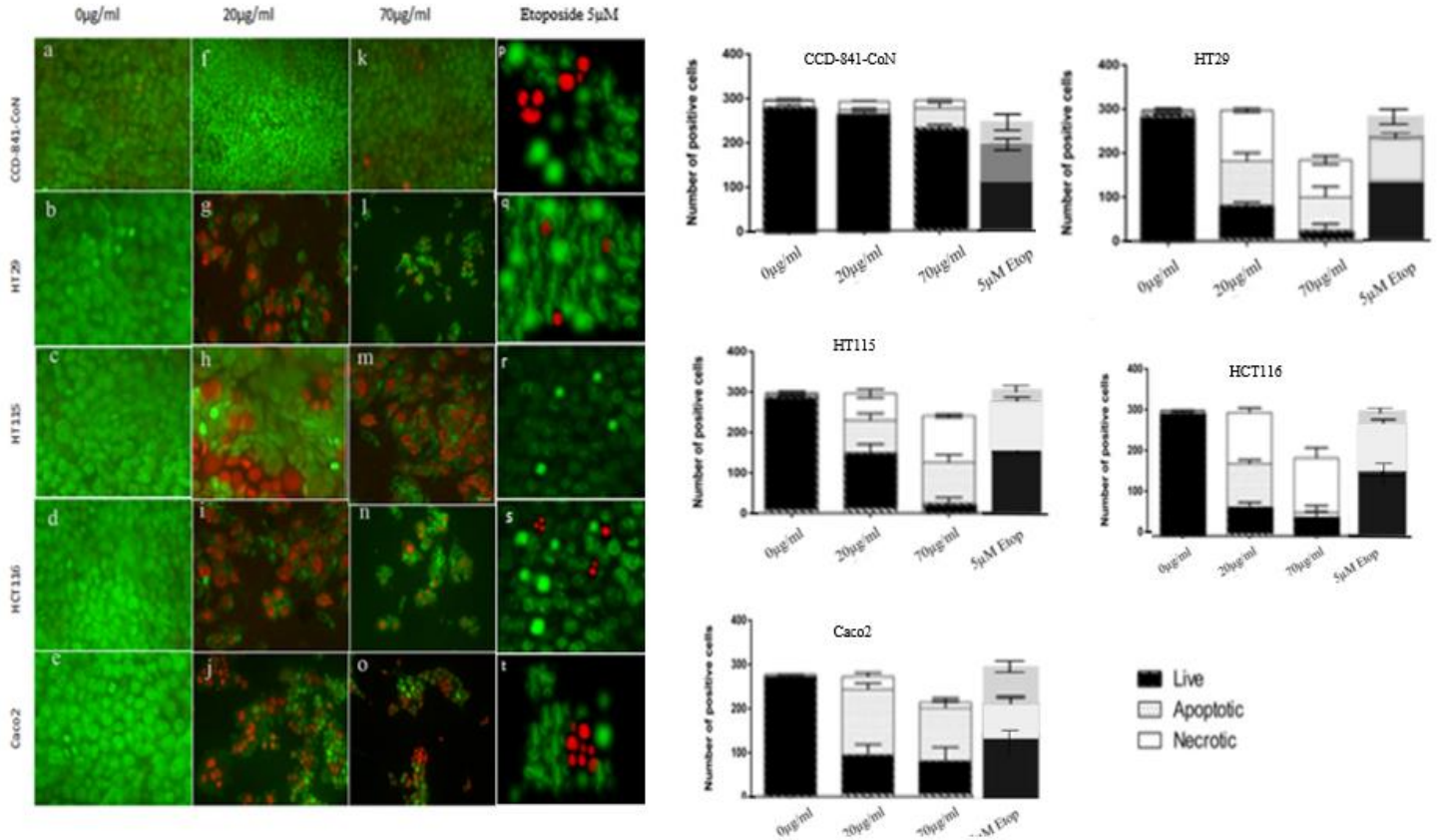


Fig 4.6: Photomicrographs of acridine orange and ethidium bromide stained cultures following treatments with 0 (a-e), 20 (f-j), 70µg/ml (k-o) ASL or 5µM Etoposide (p-t) for 24h and quantification of live, apoptotic or necrotic cells. Both 20µg/ml and 70µg/ml of ASL did not significantly induce apoptosis or necrosis in CCD-841-CoN cells (p). 20µg/ml ASL resulted in a high number of detached cells and equal numbers of necrosis (red/orange clusters) and apoptosis (fragmented green cells) in HT29, HT115 and HCT116 CRC cell lines. Caco2 cells were more susceptible to necrosis ($p < 0.01$). At 70µg/ml, very few adherent CRC cells were observed (l-o) especially in HT29 and Caco2 cell lines. HT29 (l) and HT115 (m) had 50% of cells with morphological features of both apoptosis and necrosis. HCT116 cells majority went through necrosis while a majority of Caco2 cells underwent apoptosis ($p < 0.001$) cells compared to vehicle only controls. (p-t) Cells t Values indicate mean ± SEM (n=6). Statistical significance was assessed using an one-way ANOVA.

ASL reduces motility and anchorage-independent growth of tumour cells.

To determine the effects of ASL on cell migration and invasion, the scratch (Fig 4.7a&b) and Boyden chamber assays (Fig 4.7c&d) respectively, were used. A dose of 10 µg/ml ASL had no significant effect on CCD-841-CoN cell migration, with 90% of the total scratch area being covered after 72h (Fig. 4.7a; top panel). In contrast, 10µg/ml of ASL resulted in a highly significant decrease in the proportion of the total scratch area covered after 72h in HT29 (10%; $p < 0.0001$) and HT115 cells (22%; $p < 0.0001$),

To test the migration potential, cells were plated in the upper portion of a Boyden chamber and the number of cells migrating in response to FCS were counted after 24h (Fig 4.7c). CCD-841-CoN cultures treated with the vehicle control were set at 100% migration to compare the effect of ASL treatment. Media supplemented with either 10 or 50µg/ml ASL did not significantly affect the migration of CCD-841-CoN cells (NS; $p > 0.05$). The addition of 10µg/ml ASL to cultures resulted in a reduction in migration of HT29 (33%) and HT115 (29%) compared to control values ($p <0.001$). The addition of 50µg/ml ASL reduced migration of HT29 and HT115 to 8.3% and 10.4% of control values respectively ($p <0.0001$).

To determine the ability of ASL to influence the invasive properties of cancer cells, CCD-841-CoN, HT29 or HT115 cells were plated on a layer of Matrigel in the upper compartment of a Boyden chamber (Fig 4.7d). The normal CCD-841-CoN cell line failed to invade through the Matrigel. At a dose of 10µg/ml ASL, 46% of HT29 ($p< <0.0001$) and 41.1% of HT115 ($p <0.001$) cells migrated through the Matrigel matrix. At 50µg/ml ASL, the percentage of invasion in HT29 and HT115 was reduced to 16.6% ($p <0.001$) and 22.1% ($p <0.0001$) of control values respectively.

Figure 4.7: ASL reduces migration and invasion properties at low doses *in vitro*

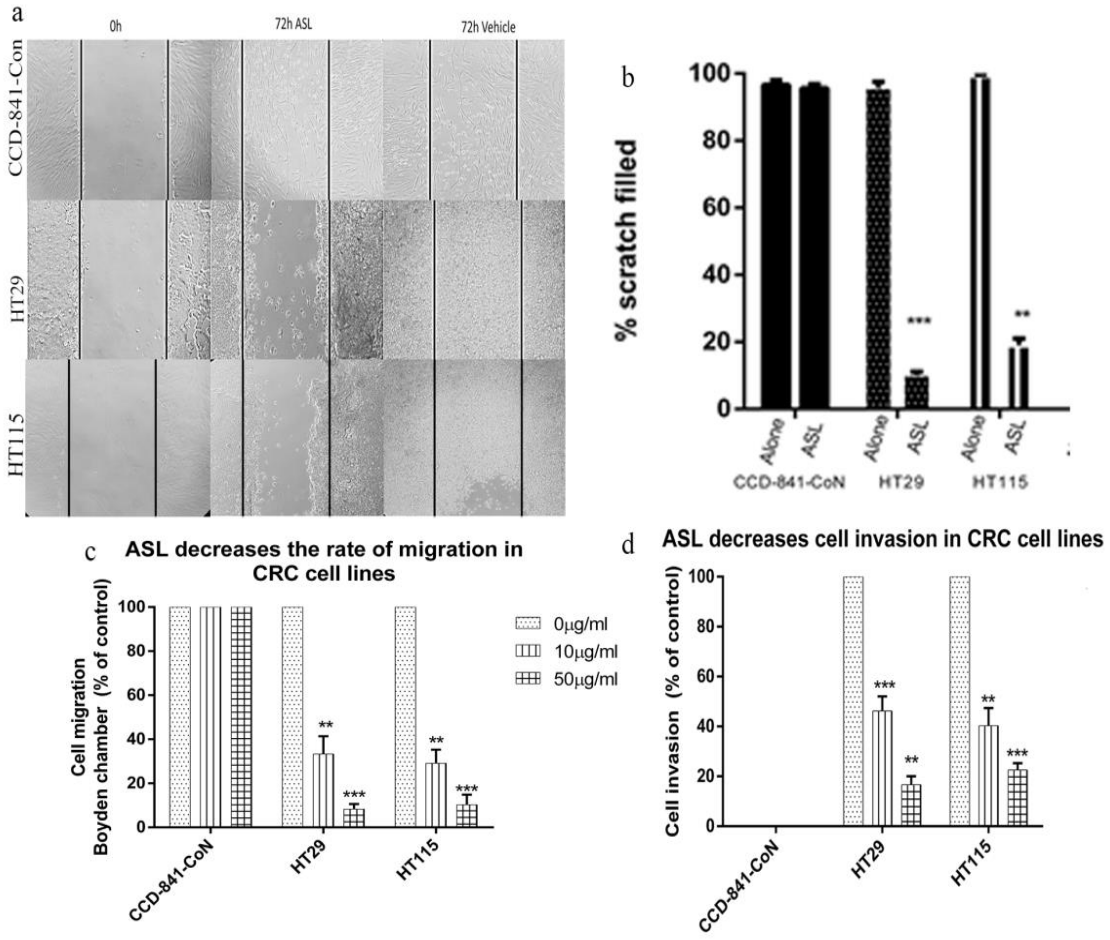


Fig 4.7: Migration and invasion of normal CCD-841-CoN and HT29 & HT115 CRC cells (a): Photomicrographs of the scratch at 0h in CCD-841-CoN (top), HT29 (middle) and HT115 (bottom), after 72h of vehicle-control or 10µg/ml ASL treated. (b): Quantification of migration in CCD-841-CoN, HT29 and HT115.A dose of 10µg/ml ASL had no effect on the migration of CCD-841-CoN after 72h..10µg/ml ASL treatments decreased the percentage of scratch migration in HT29 (12% p <0.0001) and HT115 (22% p <0.001), compared to the vehicle-control. (c) Boyden chamber migration assay of normal and CRC cells treated with 10 or 50µg/ml ASL. Migration was scored as a percentage compared to the vehicle control. Following treatment of 10 or 50µg/ml ASL, there was no significant decrease in migration rates in CCD-841-CoN. IN CRC cells HT29 and HT115, 10µg/ml ASL, migration rates dropped to 33% and 29% (p <0.001) respepctively. At 70ug/ml, migration rates of HT29 and HT115 dropped to 8.5% and 10% (p<0.0001) and 73% (**p <0.001) (d) – Matrigel invasion assay normal and CRC cells treated with 10 or 50ug/ml LSL. Invasion was scored as a percentage compared to the vehicle control. Both vehicle and treated CCD-841-CoN cells failed to invade through the matrix. At 10µg/ml 46% (p <0.0001) and 40% (p <0.001) of HT29 and HT115 cells invaded through the matrix. Doses of 70µg/ml resulted in 16.6% total invasion in HT29 (**p <0.001) and 22.1% in HT115 (p<0.0001). Statistical analysis determined using students t-test. Values represented by mean ± SEM. n=4 with 3 independent repeats.

wt and Apc$^{min+/-}$ mice tolerate the oral administration of ASL

In order to determine palatability and potential toxicity, a pilot study was performed (n = 2/group) with both *wt* and Apc$^{min+/-}$ mice which were fed either a vehicle-only control solution consisting of 10% sucrose water (vehicle-only) or vehicle containing either 0.5, 5 or 50mg/kg ASL) for a period of 5 weeks. The weights of the mice (Fig 4.8a) in addition to their water (Fig 4.8b) and food consumption (Fig 4.8c) was measured each week. Both *wt* and Apc$^{min+/-}$ mice gained weight at a similar rate, which was unaffected by the dosage of ASL administered (Fig. 4.8a). On average, both vehicle and ASL treated *wt* and Apc$^{min+/-}$ consumed 11.25 ± 2.5 grams of food (Fig. 4.8b) and 14.1 ±3.2 grams of water (Fig. 4.8c) a week.

Mice were also monitored for any change to normal behavioural habits such as grooming, nesting and reaction to humans during the study. There was no change in behaviour between any of the treatment groups (Appendix 1.1).

On completion of this oral feeding tolerance study, the major organs (liver, stomach, kidneys, lungs, heart, spleen and pancreas) were removed, weighed and a gross inspection of morphology was carried out (Fig 4.9). There were no significant differences in organ weights or gross organ morphology observed between the vehicle treated compared to ASL (0.5, 5, 50mg/kg bodyweight) treated mice.

Figure 4.8: ASL are tolerated by *wt* and Apc$^{min+/-}$ mice

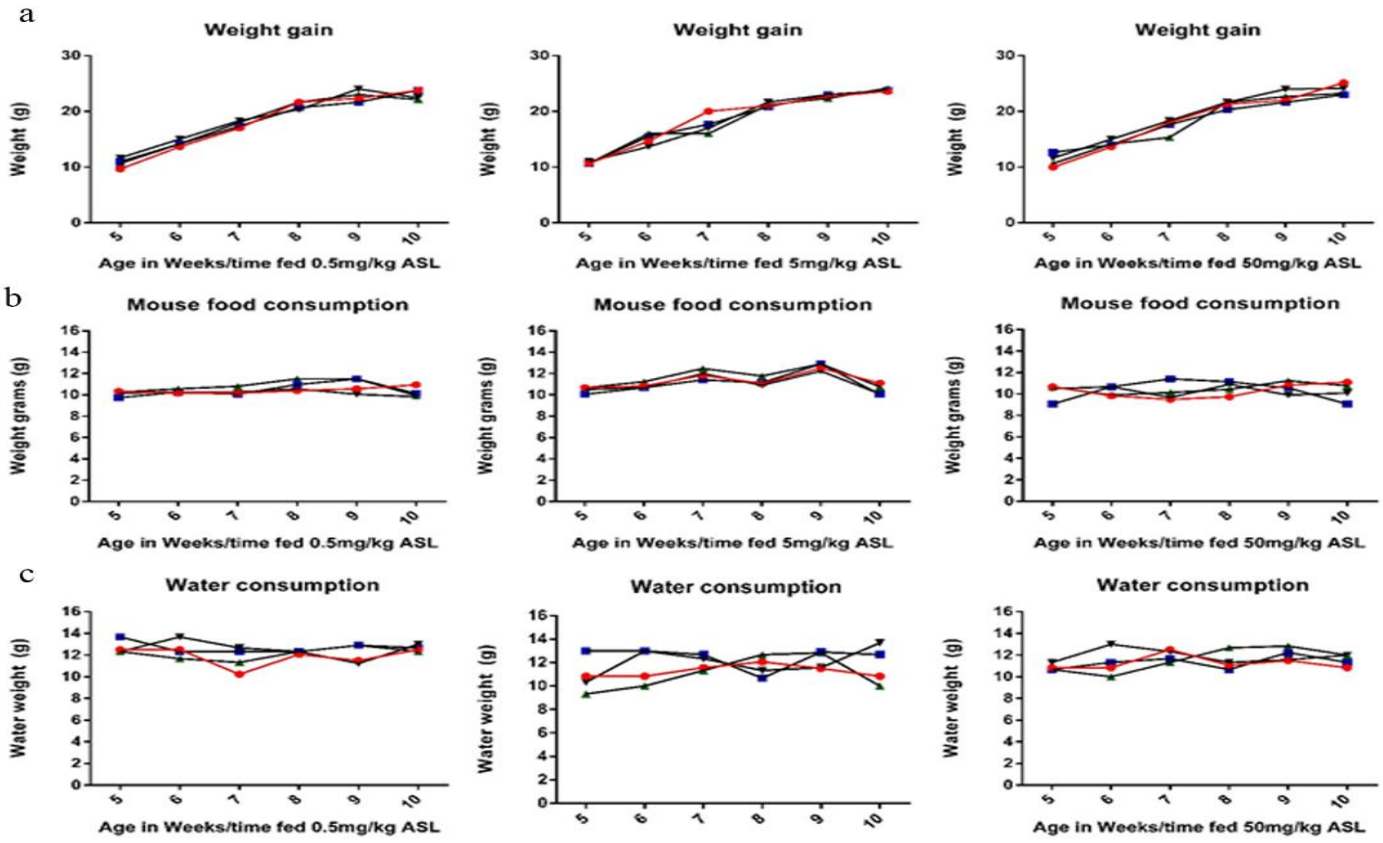
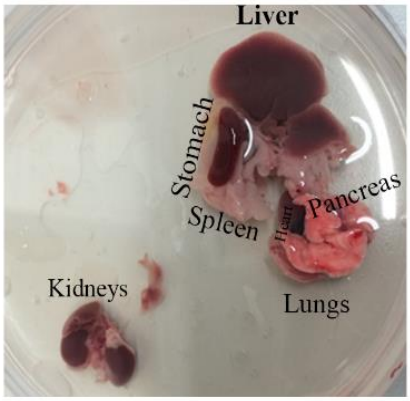


Fig 4.8: *wr* and Apc$^{min+/-}$ mice were fed vehicle or 0.5, 5 or 50 mg/kg of LSL every other day for 5 weeks and monitored closely. Mouse weights, food and water consumption was measured on a weekly basis. There were no signifcant differences in weight (a), food consumption (b) or water consumption (c) . Statistcal analysis was carried out using one-way ANOVA. Values represented by mean. n=6 per group.

Figure 4.9: Short term ASL treatment exerts no adverse effects on organ morphology or weight.

a



b

	wt vehicle	*wt ASL*	Apc$^{min+/-}$ *vehicle*	Apc$^{min+/-}$ ASL
Liver	0.74g	0.70g	0.75g	0.729g
Stomach	0.525g	0.551g	0.55g	0.52g
Kidneys (both)	0.42g	0.401g	0.44g	0.413g
Lungs	0.11g	0.10g	0.14g	0.12g
Heart	0.14g	0.12g	0.114g	0.10g
Spleen	0.18g	0.16g	0.562g	0.56g
Pancreas	0.179g	0.189g	0.19g	0.180g

Fig 4.9: wt or Apc$^{min+/-}$ mice were fed vehicle or 0.5, 5, 50mg/kg ASL for 5 weeks. There were no changes in the liver, stomach, kidneys, lungs, spleen or pancreas morphology (a) or weights (b) between treatment groups. Organs was taken from 50mg/kg but representative of all three ASL concentrations.

ASL does not affect the size or number of tumours in Apc$^{min+/-}$ mice.

The weights of *wt* and Apc$^{min+/-}$ littermate mice fed with either vehicle-only control or ASL solutions were not significantly different (endpoint: 25.2 ± 0.9g vs 22.9 ± 1.8g; NS, $p > 0.05$) and there were no differences in water or food consumption over the duration of the experiment. The gross morphological appearances of unfixed, flat-mounted ilea from *wt* mice (Fig. 4.10a top) treated with either vehicle or 50 mg/kg ASL was characterised by a flattened, uniformly smooth mucous epithelium with prominent patent blood vessels. In vehicle-only treated Apc$^{min+/-}$ mice (Fig. 4.10a; bottom left), there is clear evidence of polyp associated bleeding (arrow heads) within the ileal segment. Following treatment with 50 mg/kg ASL for 70 days, there was little evidence of bleeding from intestinal polyps (Fig. 4.10a; bottom right) in comparison to vehicle treated animals (4.10a; bottom left). The number of intestinal polyps in Apc$^{min+/-}$ mice was not significantly different following treatment with 50 mg/kg ASL for 70 days (vehicle = 48 ± 2 *vs* ASL = 45 ± 4; Fig. 4.10b; $p < 0.1$). ASL treatments also had no effect on the modal size distribution of the polyps in comparison to vehicle-only (vehicle- 4mm vs ASL 4mm; Fig 4.10c; NS, $p > 0.05$).

Figure 4.10: ASL has no effect on poylp development in the Apc$^{min+/-}$ mice

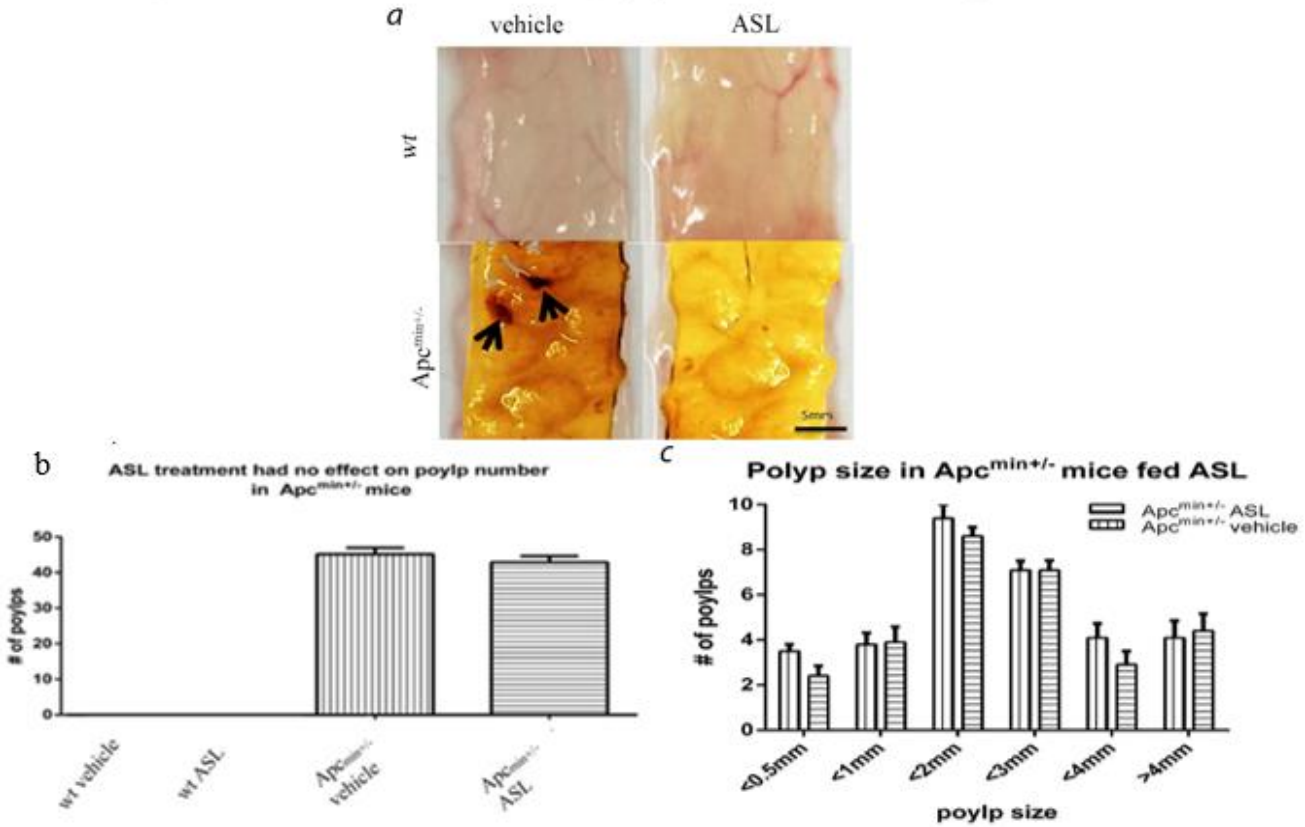


Fig 4.10: Mice (wt or Apc$^{min+/-}$) were fed ASL or vehicle-only every other day for 70 days. There were no morphological changes to ileal segments from wt mice fed vehicle-control or 50mg/kg ASL (a: top). Apc$^{min+/-}$ ileum (a: bottom) treated with vehicle-only (bottom left) showed evidence of bleeding (arrow heads) as well as numerous polyps with a diameter between 0.4mm-4mm (c). Ileal segments from Apc$^{min+/-}$ mice treated with ASL (a: bottom right) showed no evidence of intestinal bleeding and no significant change to polyp number (b: cntrl:48 vs ASL:42) or diameter (c). Values represent mean ± SEM (n=10/group). n= 10 per group.Statistical significance was determined by one-way ANOVA or a student T-tests.

ASL reduces splenic weight and the proportion of red pulp in Apc$^{min+/-}$ mice

The spleens from vehicle-control fed Apc$^{min+/-}$ mice (0.58 ± 0.2g) were significantly heavier than their *wt* littermates (0.15g ± 0.5g; Fig. 4.11a&b; p <0.0001). A dose of 50mg/mg ASL had no effect on splenic weight in *wt* mice after 70 days of treatment. However, administration of 50 mg/kg ASL for 70 days to Apc$^{min+/-}$ mice resulted in a statistically significant decrease in splenic weight (Apc$^{min+/-}$ = 0.58 ± 0.2 g vs ASL Apc$^{min+/-}$ 0.40 ± 0.4g; Fig 4.11b; p < 0.001).

Histological examination of sections from *wt* mouse spleen (Fig. 4.11c; left panel) revealed conspicuous intensely basophilic areas of white pulp; these are separated by less dense regions of red pulp, the areas that are responsible for removal of old or damaged erythrocytes. In vehicle treated Apc$^{min+/-}$ mice there is conspicuous clumping of the red pulp and the proportion of this tissue is significantly increased when compared to *wt* (middle vs left panel; Fig. 4.11c). Following treatment with 50 mg/kg ASL there was a significant reduction in red pulp size as compared with vehicle-only controls (Fig. 4.11c right vs middle panel; Fig. 4.11d).

Haematocrit levels were not significantly different after 35 days of ASL treatment between *wt* and Apc$^{min+/-}$ mice (41.2% ± 0.8 vs 40.0% ± 1.1; p <0.1). However, after 70 days of ASL administration, haematocrit levels were significantly higher in *wt* than Apc$^{min+/-}$ mice and feeding Apc$^{min+/-}$ mice with 50 mg/kg ASL resulted in a significant increase in haematocrit compared to the vehicle-only control (Fig. 4.12; vehicle = 36.0% ± 1.9 *vs* ASL = 42% ± 1.2; p < 0.05).

Figure 4.11: ASL provides splenic protection and improves haematocrit in Apc$^{min+/-}$ mice

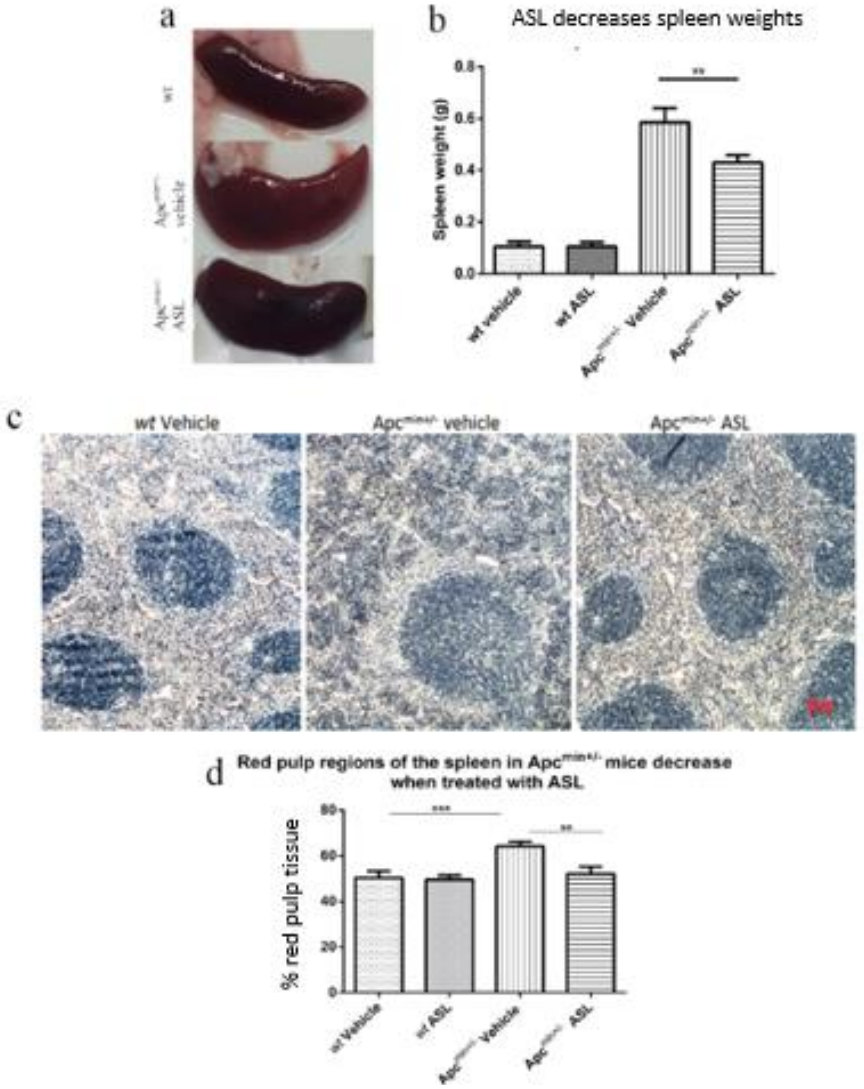


Fig 4.11: (a); Photographs of dissected spleen from a *wt* mouse and Apc$^{min+/-}$ mouse fed with vehicle-only or 50mg/kg ASL for 70 days. (b); Graph indicating the difference in spleen weights. Apc$^{min+/-}$ mice had a significantly greater weight compared to *wt* mice (*** $p<0.0001$). ASL dosing resulted in a significant decrease in splenic weights compared to the vehicle control mice (** $p < 0.001$). Splenic histology from *wt* (c top), Apc$^{min+/-}$ mice fed vehicle control-only (6c middle) and Apc$^{min+/-}$ fed ASL (6c bottom). WP = While Pulp, RP = Red Pulp. *wt* spleens are characterised by the white pulp and the loose reticular structure of the red pulp. Apc$^{min+/-}$ mice demonstrated altered splenic pathology with increase red pulp regions (haematopoietic rich tissue). ASL fed mice showed an improvement in histopathology (c.f 6c middle vs bottom) by decreasing the red pulp population (* $p < 0.05$). Values represented of ± SEM. (n=10/group). Statistical significance was determined by students t-test.

Figure 4.12: ASL improves haematocrit levels in $Apc^{min+/-}$ mice.

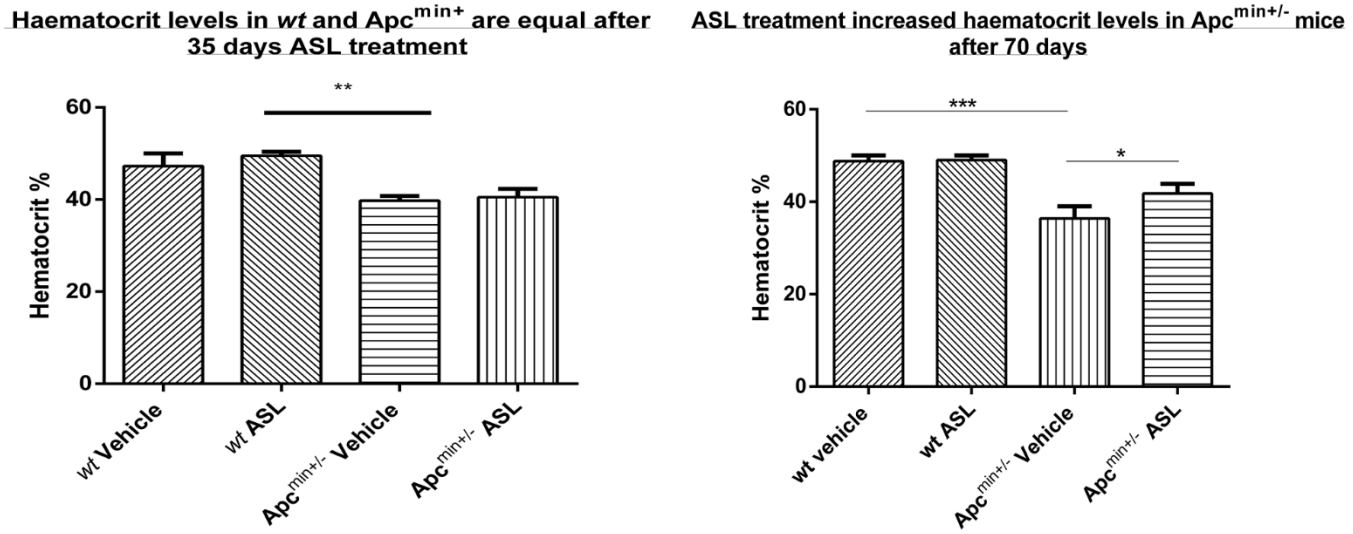


Figure 4.12: Haematocrit measurements from *wt* or $Apc^{min+/-}$ mice fed vehicle control or ASL after 35 days (a) or 70 days (b) of treatment. After 35 days of ASL treatement there were no significant differences in haematocrit levels between *wt* or $Apc^{min=/-}$ mice and no difference between treatment groups. After 70 there was a significant reduction in haematocrit levels in $Apc^{min+/-}$ compared to *wt* mice (48.9 ± 0.5 vs 36.0 ± 1.9 $p < 0.0001$). ASL treatments for 70 days resulted in an increase in haemocrit levels in $Apc^{min+/-}$ compared to vehicle control mice (36.0 ± 1.9 vs 42 ± 1.2 $p < 0.01$). Statistical analysis determined using students t-test. Values represented by mean ± SEM. n=10 per group.

4.4 Discussion

The ability of chemotherapeutic agents to distinguish between normal tissue and cancerous cells is a highly desirable trait, as it may mitigate against the common side effects associated with this toxic therapeutic (McQuade et al., 2014) such as hair loss and immunosuppression (Burish, 1988).

Here we investigated the effects of a 94% pure preparation of ASL on two normal cell lines (CCD-841 and MRC5) and 5 CRC cell lines (HT29, HT115, Caco2, HCT116 and LS180) *in vitro*. Colonic epithelial (CCD-841-CoN) and lung fibroblasts (MRC5) treated with ASL showed no evidence of toxicity as evidenced by maintenance of their viability at doses ranging between 0.01 – 100µg/ml. However, at doses of 20µg/ml and above, ASL potently reduced cell viability in all the CRC cells examined. Although the cytotoxic potency of SL mixtures has been reported against cancer cell lines previously (Chen et al., 2006a, Fu et al., 2008, Shao et al., 2012, Ribeiro et al., 2015), this is the first time an anti-cancer effect has been reported from a purified and well characterised ASL on a range of cell lines from the same tissue of origin (i.e. colorectal cancer cells).

Interestingly, ASL exhibit a differential effect in normal compared to CRC cells *in vitro*, with a dose-dependent cytotoxic response only observed in the five CRC cell lines across the range of doses examined. A differential effect has been reported in previous studies with SL mixtures of uncertain purity (Fu et al., 2008) where SL were cytotoxic to both pancreatic and liver cancer cell lines but with no demonstrated toxicity to a normal cell line. However, in the aforementioned study non-adherent, circulating blood monocytes (PBMC) were used as a control for adherent transformed cells making conclusions from this study difficult to interpret. Therefore, we believe this to be the first directly comparable description of the specificity of a pure preparation of ASL that uses a normal adherent cell line from the same tissue of origin as the transformed CRC cells.

It has been hypothesised that SL have the ability to intercalate into the cytoskeleton of cells resulting in membrane disruption. Changes in cell morphology consistent with disruption of membrane cytoskeletal protein distribution have previously been shown to occur with SL doses as low as 30µg/ml in pancreatic H7402 and lung cancer A549 cell lines (Chen et al., 2006a). Our study demonstrates that single congener ASL at low concentrations (40µg/ml) induce cell rounding, cytoplasmic condensation and cell detachment (Fig 4.4) in all the CRC cancer cell lines tested, with the adenocarcinoma cell lines HT29 and Caco2 being most susceptible. At doses of 70 - 100µg/ml, ASL causes a significant increase in the numbers of detached cells with all cells in the supernatant from treated cultures showing morphological features consistent with apoptosis or necrosis (Fig 4.5). Although the difference between live and dead cells, are easily seen, it is harder to distinguish between apoptosis and necrosis in this assay. To be able to better compare the data, future studies would benefit from the addition of both an apoptosis (Apoptolidin (Chong *et al.,* 2016)) and necrosis (Chrysophanol (Lu *et al.,* 2010)) inducer. Future studies could also use a flow cytometer to analysis apoptosis or necrosis after SL treatments. This may prove a better non-bias approach to quantification of cell death mechanisms (Liu *et al.,* 2015).

BS used at the doses described in our study, induce a reduction of tension at the interfacial region of the bilayer resulting in phospholipid dehydration which affects lipid stability and ultimately results in cell death (Fracchia et al., 2012, Shah et al., 2005). To assess cell death mechanisms caused by ASL, ethidium bromide/acridine orange staining was carried out allowing the morphological identification of the type of cell death. We have conclusively demonstrated that ASL induces both apoptosis and necrosis in these cancer cell lines in a dose-dependent manner. The ability of ASL to induce either apoptosis or necrosis may be cell line specific as high proportion of apoptotic cells have been reported following the addition of diacetylated LSL to liver (H7402) and lung cancer (H7402) cell

cultures, while necrosis is primarily observed in a pancreatic carcinoma cell line (HPAC) treated with SL mixtures, LSL or methyl ester derivative SL (Shao et al., 2012).

An important characteristic of malignant growth is the ability of tumour cells to leave their restricted compartment and gain access to blood vessels to initiate the first phase of metastasis (Hanahan and Weinberg, 2011). The movement of cells across tissues therefore plays an important role in this progression thus highlighting the need for an agent that can counteract the migratory and intravasative properties of CRC cells (Dianzani et al., 2014). It has been postulated that the amphiphilic properties of SL permit their incorporation into the mammalian cellular membrane disrupting cellular functions such as proliferation and migration (Fracchia et al., 2015). We assessed the ability of ASL to inhibit migration of the CRC cell lines HT29 and HT115. At a low doses (10µg/ml) of ASL there was no effect on normal colonic epithelial cells (CCD-841-CoN) and scratch coverage after 72 hrs. However, when the same dose of ASL (10µg/ml) was applied to HT29 and HT115 cultures the total percentage area of scratch covered was reduced. An inhibition of migration induced by SL (as measured in the scratch assay) has only been documented in one other study (Ribeiro et al., 2015) where 5µg/ml of a 93% pure, diacetylated LSL resulted in significantly reduced migration of MDA-MB-231 breast cancer cells.

The use of both migration and invasion assays incorporating the Boyden chamber are widely used to test candidate chemotherapeutics with the HT29 and HT115 cell lines commonly employed to test anti-invasive properties of potential chemotherapeutics *in vitro* (Li and Zhu, 1999). Currently the only studies investigating the potential anti-metastatic properties of BS are limited to the inhibitory effects on *in vitro* invasion mediated by the surfactant produced by *Baccillus subtilis* in the breast cancer cell lines MCF and MDA-MB-231 (Park et al., 2013). Addition of 10µM (equivalent to approx.

660µg/ml of LSL in this study) of biosurfactant had the ability to reduce the migration of MCF-7 and MDA-MB-231 through an extracellular matrix by 68% and 84% respectively. The uncharacterized surfactants used in this study also reduced the colony forming ability of both cell lines by 70% and 61%. To investigate the migration inhibitory properties of ASL, HT29 and HT115 cells were plated on a porous membrane and allowed to migrate through a septum in the chamber below. ASL had no effect on the migration of normal colonic CCD-841-CoN cells, however, migration was significantly decreased in HT29 and HT115 cultures (by 65% and 72% with 10µg/ml and 86% and 81% with concentrations of 50µg/ml ASL). A modification of this method involving placing a layer of matrigel on the porous membrane can measure the ability of cancer cells to invade through a matrix in response to a stimulus (Krishnamachary et al., 2003). This *in vitro* model mimics the action of cancer cells invading through and metastasising through the body, a key step in the hallmarks of cancer (Pietras and Östman, 2010). ASL dose dependently reduced the invasion of HT29 and HT115 through an extracellular matrix with doses as low as 10µg/ml. This is the first time that inhibitory properties of ASL on tumour cell invasion have been reported. In addition, differential effects of BS congeners is a common phenomenon, previously observed when comparing the antibacterial effects of rhamnolipids, which can be produced as mono or di-rhamnolipids (Martin et al., 2016). For instance, di-rhamolipids produce a more potent antibacterial effect against *Burkholderia thailandensis* biofilm formations (De Rienzo and Martin, 2016). Similarly, mono-rhamnolipid congeners show enhanced anti-cancer potential against HL-60, BV-173, SKW-3 and JMSU-1 cells *in vitro* in comparison to the di-rhamnolipids (Christova et al., 2013) .

The anti-tumour activities of SL preparations are unclear, as in a study by Li and co-workers, an intragastrically administered SL mixture, of an undisclosed composition, was shown to reduce the size of tumours in a murine HeLa xenograft model (Li et al., 2016)

whereas a purified form of LSL exaggerated the growth of neoplasm along the intestinal tract and increased intestinal blood loss in the $Apc^{min+/-}$ mouse (Chapter 3 and Callaghan et al., 2016).

$Apc^{min+/-}$ mice develop intestinal adenomatous neoplasms (polyps) and animals typically present with enlarged spleens and reduced haematocrit by 4 months of age (Yekkala and Baudino, 2007). This is an acute model with a life span of <150 days, where the primary cause of death is not directly attributable to the development of numerous polyps but rather as a result of extensive intestinal bleeding and anaemia (Hinoi et al., 2007).

When administered orally, topically or via *i.v.* injection SL mixtures are well tolerated (Ikeda et al., 1986). Similarly, in our study, both *wt* and $Apc^{min+/-}$ mice tolerated the oral administration of relatively pure single congener ASL (this Chapter) with no measurable gross anatomical or behavioural differences noted. Post mortem analysis also revealed no effects in systematic organ measures after ASL treatment. However, oral feeding of ASL to $Apc^{min+/-}$ for 70 days resulted in a reduction of spleen size and significantly increase in haematocrit, consistent with decreased intestinal bleeding and improvement in the associated anaemia characteristic of this model (Moser et al., 1990). This is a potentially significant finding, as rectal bleeding and anaemia are reported in over 30% of CRC patients and it is a contributing factor in reduced life-span in both humans (Ronnekleiv-Kelly and Kennedy, 2011) as well as $Apc^{min+/-}$ mice . In humans, laser ablation encourages coagulation of tumours demonstrating significant blood loss and this technique has shown to be effective after 2 - 5 treatments with a success rate of 80% (Kimmey, 2004). However recurrent bleeding episodes result in surgical intervention in 2 - 15% of patients (Rao et al., 2005). The oral administration of a well-tolerated non-toxic, pro-thrombotic agent to reduce intestinal blood loss in patients with haemorrhagic colo-rectal tumours may be a useful addition to the therapeutic treatment of these conditions. As mentioned in chapter

three, this chapter would also benefit from the addition of controls such as Celebrex, a cyclooxygenase-2 inhibitor which can reduce the size of polyps in this model. This would help complement the data making conclusions on the ASL more scientifically conclusive. As seen in the data, the 96% pure C18:1 ASL had no effect on polyp number. Future studies could look at other ASL congeners or a mixture of C18:1-3 ASL congeners to see if it would improve the biological activity *in vivo*.In conclusion, the purified ASL mixture we report in this study differentially effects non-transformed in comparison to colorectal cancer cell lines, resulting in a significant and dose-dependent decrease in their viability, migration and anchorage-independent growth characteristics. While ASL does not change either the size or number of intestinal polyps in $Apc^{min+/-}$ mice, both spleen size and tumour-associated bleeding was apparent. This warrants further investigation of this orally available BS as a chemotherapeutic for delaying disease progression in pre-cancerous colorectal neoplasms.

Overall, when comparing the effects of LSL in chapter three and the ASL in this chapter – it is easy to see that ASL provides a more desirable biological effect. Unlike LSL – ASL demonstrates the ability to differentiate between normal and cancerous cell lines, inflicting a response only to the latter. The mechanisms behind how this phenomenon occurs is yet unknown. It can be hypothesised that ASL is fast acting compared to LSL and targets fast cancer proliferating cells. This could be address using time scale tests and comparing how fast ASL vs LSL affects the cells. The difference in biologically activity could also be associated with the ability of SL to enter the cell. These questions can be explored using mechanistic *in vitro* studies.

Chapter 5

Investigation of the mechanism of SL-induced death in HT29 CRC cells

5.1 Introduction

Biosurfactants in general and SL mixtures in particular, have been well documented to inhibit tumour cell growth *in vitro*, however the mechanisms of action underlying this effect is still unclear. The first insight into the inhibitory properties of SL mixtures was observed following the treatment of H7402 liver cancer cells, which resulted in an increase in the percentage of cells arresting in the G1 and S1 phases (Chen et al., 2006a). This group also reported an influx of Ca^{2+} released into the supernatant and an increase in caspase-3 levels after SL treatment *in vitro*. They hypothesised that SL treatment in cancer cells causes an increase of intracellular Ca^{2+} which results in the arrest of cells in the G1 and S1 phase of the cell cycle, inducing apoptosis *via* the capsase-3 pathway (Chen et al., 2006a).

It was initially postulated that the mechanism of action of BS induction of cell death is *via* triggering a non-specific, detergent-like effect that causes the formation of holes in the cell membrane (Heerklotz et al., 1997). This concept was developed in studies showing the ability of BS to cause destabilisation of lipid-rich clusters in the bilayer of the cell membrane, leading to a disruption in lamellar structure and leakage of cytoplasmic material (Heerklotz, 2001, Heerklotz and Seelig, 2007). This membrane disrupting activity has previously been shown for preparations of both non-ionic surfactants (Groot and Rabone, 2001) and BS produced by *Bacillus subtilis*, and is believed to be mediated by their surfactant properties (Rodrigues et al., 2006).

Due to their lipid structure, it has been hypothesised that BS can be inserted into the outer lipid layer of a cell causing an increase in lateral pressure and disruption to the cell membrane (Heerklotz, 2001). This mechanism of action has been established with surfactan (a lipopolysaccharide), using a vesicle membrane to model the characteristics of a human cell membrane which allows the analysis of membrane incorporation, membrane leakage and cell lysis using fluorescent dyes (Heerklotz and Seelig, 2007). The

model has also been used to investigate the BS Trehalose, which was shown to incorporate into cell membranes confirming that BS can self-insert into the phospholipid bilayer causing dehydration of the phospholipids, thus disrupting protein conformations and affecting cell transport and energy production (Ortiz et al., 2009, Heerklotz, 2008).

The biological action of surfactant is dependent on its concentration and saturation, with low concentrations (<100µg/ml), penetrating easily into the membrane and having no deleterious effects on the membrane integrity but affecting cell adhesion and migration. At concentrations between 100 - 1000µg/ml, surfactants such as triton x-100 cause pore formation on the cell surface and at concentrations above 1mg/ml), surfactants cause complete permeabilization of the cell (Carrillo et al., 2003).

Mechanistic studies revealed that non-ionic surfactants disrupt the natural distribution of lipid rafts in the cell membrane resulting in changes in cell shape, structure, composition and size (Heerklotz, 2002). This phenomenon is accompanied by disruption in the natural signalling pathway of the cell causing changes to viability, as well as adhesion (Huang et al., 2006) and the activation of cell death (Bang et al., 2005). An *ex vivo* study employing isolated neural stem cells from the subventrical zone (SVZ) of mice demonstrated that di-rhamolipids (a type of glucolipid) have a dose-dependent effect on cell viability (Stipcevic et al., 2013). The authors hypothesized that rhamolipids induce this response by interfering with the lipid raft domain, disrupting cell-signalling pathways (Stipcevic et al., 2013).

Lipid rafts are micro-domains present in the cell membrane bilayer which are heterogeneous molecular complexes made up of tightly packed lipids (cholesterol and sphingolipids) and can include proteins. They play a vital role in physiological processes such as polarization, signalling (Simons and Toomre, 2000), trafficking, adhesion, sorting and regulation of cell death mechanisms (Simons and Ikonen, 1997, Brown and London,

1998). Little is known about the effects cell transformation has on lipid rafts, however it is thought that they undergo structural changes resulting in fluctuations in protein expression and alterations to raft linkage to the cytoskeleton rendering the rafts unstable (Staubach and Hanisch, 2011).

Lipid rafts can be visualised microscopically using the cholera toxin subunit B (CTxB) (Harder et al., 1998) which can be fluorescently tagged. In culture, lipid rafts can be seen as a fluorescent coating surrounding the outer layer of the cell membrane. Under cellular stress – such a drug therapy or the development of pathological diseases, an increase in the intensity of CTxB is observed (Allen et al., 2007). In the initial stages, small clusters are evident at the polar cap of the cells. Prolonged stress can result in the visible formation of several clusters. These clusters have been linked to the activation of receptors and it is hypothesised that a majority of cell signalling occurs in these clusters in the lipid rafts (Simons and Ehehalt, 2002). These clusters have been seen in HT9 cells in response to cisplatin (Lacour et al., 2004) and immune cells in reaction to IgE production causing allergies (Sheets et al., 1999).

Studies have also shown that disruption to lipid rafts can result in changes to tight junctions which result in a decrease in cell migration and invasion rates in cancer cells (Murai, 2011). It has also been shown that lipid raft changes cause alterations to the mitochondrial membrane potential of cells (MMP) (Gajate and Mollinedo, 2014) and can result in an increase of reactive oxygen species (ROS) (Turrens, 2003), resulting in cell death (Simon et al., 2000). Currently, research aimed at the targeting of lipid raft domains in cancer cells has attracted great interest (Mollinedo and Gajate, 2010) with several potential chemotherapeutic agents targeting multiple myeloma, leukaemia (Gajate and Mollinedo, 2007) and prostate cancer (Zhuang et al., 2005) respectively, being trialled. It is also believed that the targeting of lipid rafts may help decrease the phenomenon of

multidrug resistance that is currently observed with conventional chemotherapeutics (Hendrich and Michalak, 2003).

5.2 Hypothesis

The treatment of HT29 CRC cells with SL results in the redistribution of lipid rafts, which disrupts the tight junction distribution and increases intracellular ROS production.

Aims and Objectives:

1) Determine the differential effect of SL on lipid raft disruption between "normal" colonic (CCD-841-CoN) and CRC HT29 cell cultures *in vitro.*
2) To determine if ASL and LSL interrupt ZO-1 distribution in CCD-841-CoN and HT29 cells.
3) Determine if SL treatment results in a change to Mitochondrial Membrane Potential levels and Reactive Oxygen Species production in CCD-841-CoN and HT29 cells.

5.3 Materials and methods

Lipid rafts

A total of $3x10^4$ of CCD-41-CoN or HT29 cells were seeded onto a 10mm coverslip (Agar Scientific; Stansted: Essex, UK), incubated overnight in a 24 well plate to attach. Media was replaced with serum free media and further incubated for 24h. Vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10 or 50µg/ml final

concentration) were added to the cell lines for 24h. Cells were then washed with ice cold PBS and incubated with 200µl of fluorescent CT-B conjugate (1µg/ml; Invitrogen; Paisley, UK) for 20 minutes at 4ºC in the dark. After aspiration of the liquid, the cells were washed three times with ice cold PBS and subsequently fixed in 10% neutral buffered formalin (Sigma-Aldrich; Dorset, UK) for 15 minutes at 4ºC in the dark. Coverslips were mounted on to a slide using histamount and visualised immediately by florescence microscopy (Axio Scope 1, Zeiss, Germany) using a 488nm filter at 40x objective magnification.

ZO-1 expression

A total of $3x10^4$ of CCD-41-CoN or HT29 cells were seeded onto a 10mm coverslip (Agar Scientific; Stansted: Essex, UK), incubated overnight in a 24 well plate to attach. Cell growth media (see above) was replaced with serum free media and the cells further incubated for 24h. Vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10 or 50µg/ml final concentration) were added to the cell lines for 24h. The cells were subsequently washed with ice cold PBS and fixed with neutral buffered formalin (Sigma-Aldrich; Dorset, UK) for 20 minutes at room temperature. A ZO-1 antibody (Invitrogen; Paisley, UK) was added overnight using a 1:1000 dilution in 5% BSA (Sigma-Aldrich; Dorset, UK) at 4ºc. Cells were washed with ice cold PBS several times. Cells were stained with Hoechst (Invitrogen; Paisley, UK) for 10 minutes in the dark and washed with ice-cold PBS. Coverslips were mounted on to a slide using histamount and visualised immediately by florescence microscopy (Axio Scope 1, Zeiss, Germany) using a 594nm (ZO-1) and 461nm (Hoechst) filter at 40x magnification.

Measuring Mitochondrial Membrane Potential:

MMP quantification was carried out as previously described (Mortiboys *et al*.,2008). A total of $1x10^3$ of CCD-41-CoN or HT29 cells were seeded into a 96 well plate (Nunc

Thermos scientific, UK) and incubated overnight to attach. Media was replaced with serum free media and further incubated for 24h. Cells were treated with either Vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10 or 50µg/ml final concentration) were added to the cell lines for 24h. Cells were incubated with 1µM dihexyloxacarbocyanine iodide (DiOC6) (Sigma-Aldrich; Dorset, UK) for one hour at room temperature and washed with ice cold PBS. Cells were detached from the plate using 0.25% trypsin (Gibco Invitrogen; Paisley, UK) and re-suspended in 100µg/ml of PBS. Fluorescence intensity was read in FLUOstar Omega plate reader (BMG lab tech; Aylesbury, UK) using filter 488nm.

In order to compare results between cell lines and experiments, intensity was normalised to cell number. This was carried out by plating out $1x10^3$ of CCD-41-CoN or HT29 cells in a parallel plate. Cells were treated with either vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10 or 50µg/ml final concentration) were added to the cell lines for 24h. Cells were incubated with 1µm ethidium homodimer fluorescent dye (Sigma-Aldrich; Dorset, UK) for one hour at room temperature and washed with ice cold PBS. Cells were detached from the plate using 0.25% trypsin (Gibco Invitrogen; Paisley, UK) and re-suspended in 100µg/ml of PBS. Fluorescence intensity was read in FLUOstar Omega plate reader (BMG lab tech; Aylesbury, UK) using filter 544nm (Mortiboys *et al*.,2008).

Cellular Reactive Oxygen Species Detection Assay:

A total of $1x10^3$ of CCD-41-CoN or HT29 cells were seeded into a 96 well plate (Nunc Thermos scientific, UK) and incubated overnight to attach. Media was replaced with serum free media and further incubated for 24h. Cells were treated with either. Vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10, 20, 40, 60, or 80µg/ml final concentration) were added to the cell lines for 24h or H_2O_2 (0.1mM;

Sigma-Aldrich Company Ltd, Dorset, UK) for 24h. Cells were incubated with 10µM H_2-DCFDA (Sigma-Aldrich; Dorset, UK) for one hour at room temperature and washed with ice cold PBS. Cells were trypsinized from the plate using 0.25% trypsin (Gibco Invitrogen; Paisley, UK)) and re-suspended in 100µg/ml of PBS. Fluorescent intensity was read in FLUOstar Omega plate reader (BMG lab tech; Aylesbury, UK) using filter 488nm.

In order to compare results between cell lines and experiments, intensity was normalised to cell number. This was carried out by plating out $1x10^3$ of CCD-41-CoN or HT29 cells in a parallel plate. Cells were treated with either vehicle-only solutions (ASL: PBS or LSL: 0.01% DMSO in PBS), ASL or LSL (at 10 or 50µg/ml final concentration) were added to the cell lines for 24h. Cells were incubated with 1µm ethidium homodimer fluorescent dye (Sigma-Aldrich; Dorset, UK) for one hour at room temperature and washed with ice cold PBS. Cells were detached from the plate using 0.25% trypsin (Gibco Invitrogen; Paisley, UK) and re-suspended in 100µg/ml of PBS. Fluorescence intensity was read in FLUOstar Omega plate reader (BMG lab tech; Aylesbury, UK) using filter 544nm (Mortiboys *et al*.,2008).

5.4 Results

Treatment with SL results in a disruption of lipid raft structures *in vitro*

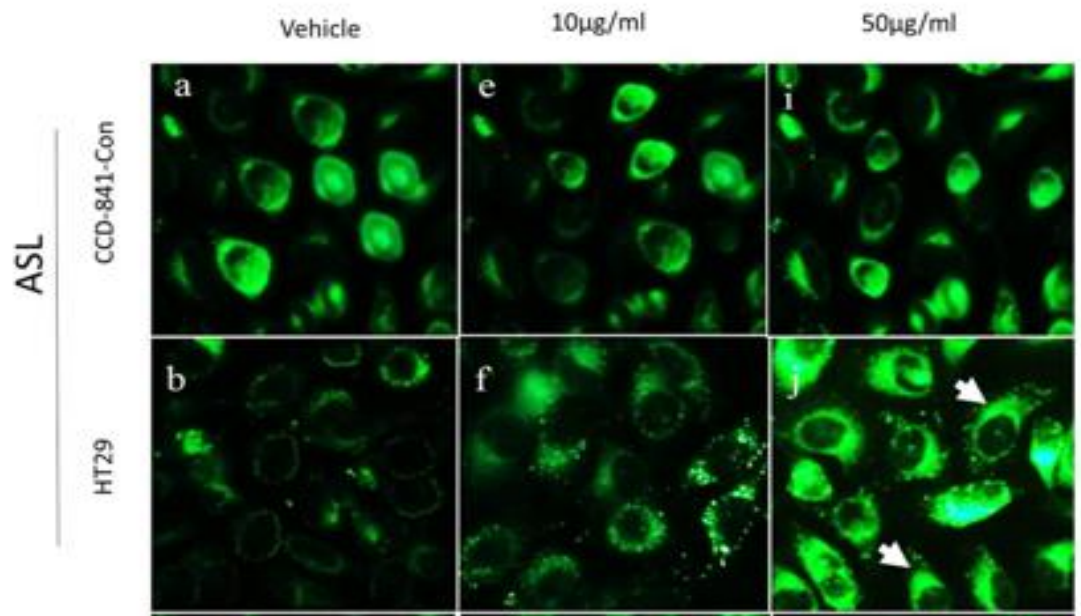
To explore the possible mechanisms for the cell death inducing effects of SL (as shown in Chapter 3 &4), we examined the effects of SL on lipid raft structure in HT29 CRC cells *in vitro*. We examined the cell membranes of HT29 cells, via fluorescent imaging, exposed to 10 and 50µg/ml of ASL and LSL as these concentrations were shown (in

Chapters 3 & 4) to have an effect on both CCD-841-CoN and HT29 cells without causing changes to cell structure. Visualisation of lipid rafts was then performed by probing treated cultures with cholera toxin subunit B (CT-B) a well described marker of lipid rafts in (Fig 5.1) and subsequently examining the cultures with fluorescence microscopy. The normal distribution of lipid rafts in colonic epithelial cells CCD-841-CoN (Fig. 5.1a&e) and HT29 (Fig. 5.1b&d) CRC cells reveals a normal distribution of lipid rafts around the outer compartment of the membrane with no specific areas containing fluorescently stained clusters visible.

Treatment of CCD-841-CoN cells with either 10µg/ml or 50µg/ml of ASL (Fig 5.1e, i) did not result in any conspicuous changes in the structure or distribution of the lipid raft membranes. However, a dose of 10µg/ml of ASL resulted in a change in the distribution of lipid raft in HT29 CRC cells via the formation of clusters evident by an increase in fluorescence intensity (Fig 5.1f) which further increased when treated with a dose of 50µg/ml ASL (Fig 5.1j white arrows).

In comparison, LSL caused significant visible changes to the disruption of lipid rafts in CCD-841-CoN cells from 10µg/ml (Fig 5.1g). Cells presented with high CT-B positivity showing activated raft clusters disrupted throughout. However, examination of the normal CCD-841-CoN cells after 50µg/ml LSL treatments were difficult due to a large proportion of areas devoid of cells (Fig 5.1h). 10µg/ml of ASL demonstrated no significant changes to lipid raft morphology or disruption in HT29 cells compared to the vehicle control (Fig 5.1h). At 50µg/ml LSL there was a change in lipid raft disruption observed in HT29 cells with a small number of cells showing signs of raft clustering (Fig 5.1).

Figure 5.1 Lipid raft disruption after SL application *in vitro*



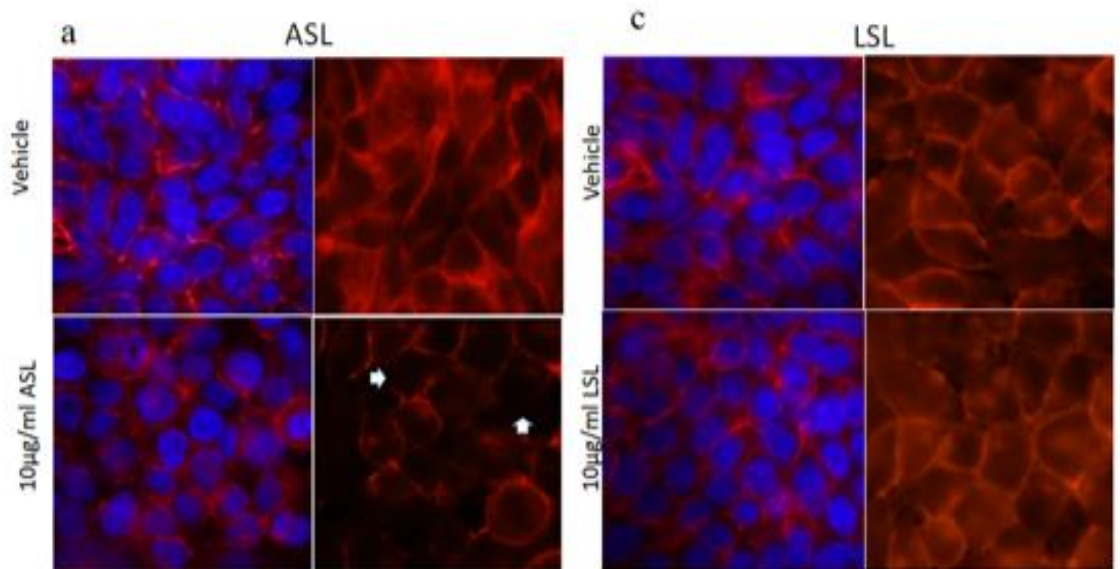
SL disrupts gap junctional morphology in HT29 CRC cells

To examine the effects that SL have on tight junction permeability, HT29 cells were treated with SL and later ZO-1 expression was examined by immunofluorescence staining (Fig 5.2a, top panel). After treatment with ASL at either 10µg/ml (Fig 5a, middle panel) or 50µg/ml (Fig 5a, bottle panel), a depletion of intracellular ZO-1 staining was observed within the HT29 monolayer (Fig 5b).

Treatments of HT29 monolayer cultures with 10µg/ml LSL (Fig 5c middle panel) caused no obvious change in the intensity or distribution of ZO-1 expression when compared to the vehicle control (top panel). At a concentration of 50µg/ml (bottom panel), intracellular depletion of ZO-1 was observed.

Quantification of the florescent intensity observed in the HT29 cells was obtained by analysing the photomicrographs on image J (Fig 5b, d). The treatment of HT29 with 10µg/ml of ASL resulted in a 32% decrease in ZO-1 expression ($p < 0.01$) which decreased to 49% after 50µg/ml ASL treatment. Under the same conditions, LSL did not influence the ZO-1 intensity after 10µg/ml ($p > 0.05$) however a significant decrease in intensity was observed with the 50µg/ml treatment ($p < 0.05$).

Figure 5.2 SL alter ZO-1 expression in HT29 cells *in vitro*



SL dose-dependently reduce MMP expression in HT29 cells

Mitochondria plays a vital role in the cell physiology and is a key indicator in the health status of the cell. CCD-841-CoN or HT29 cells were treated with various concentrations of ASL or LSL. MMP production was quantitated by measuring the fluorescent intensity emitted by the cells labelled with DiOC6, a dye which is used to detect MMP in cells. The fluorescence values were corrected for cell number.The treatment of CCD-841-CoN cells with ASL resulted in a significant decrease in the fluorescent intensity at

concentrations exceeding 60µg/ml (Fig 5.3a $p < 0.05$). At doses of 40 - 80µg/ml ($p<0.001$), ASL induced a dose-dependent decrease in the fluorescent intensity of HT29 CRC cells (Fig 5.3b $p<0.001$). LSL caused a reduction in the fluorescent intensity at concentrations as low as 10µg/ml (Fig 5.3c $p<0.05$) and dose-dependent decreases in fluorescent intensity were observed up to 80µg/ml ($p<0.001$). Significant reductions in the fluorescent intensity of HT29 cells were observed following exposure to LSL concentrations of 60µg/ml and above (Fig 5.3d $p<0.001$).

Figure 5.3 SL reduces MMP production in HT29 cells *in vitro*

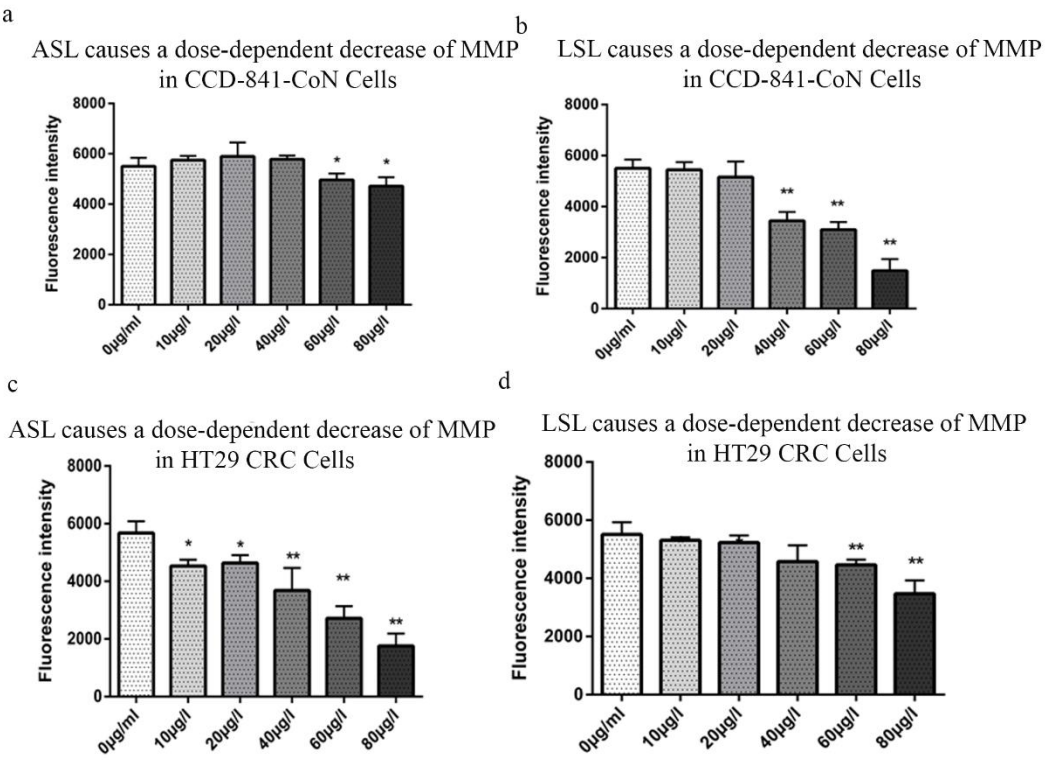


Fig 5.3: Quantification of MMP production caused by ASL and LSL in CCD-841-CoN and HT29 cells via fluorometric analysis. a; A significant decrease in MMP production was observed at 60µg/ml (* $p<0.01$a) and 80µg/ml (* $p<0.01$a) in CCD-841-CoN cells treated with ASL. However a significant reduction in MMP production was observed from 40µg/ml ASL in HT29 (** $p<0.001$b) with a dose dependent increase up to 80µg/ml (** $p<0.001$). However LSL reduced MMP production from 10µg/ml in CCD-841-CoN (* $p<0.01$c) but only induced a dose dependent decreased in MMP production after 60µg/ml in the CRC HT29 cell line (** $p<0.001$d). Graphs representative of mean ± SEM. Data representative of n=3 with three independent replicates. Statistical significance was determines using a students t-test and one-way ANOVA. * $p<0.01$, **$p<0.001$, *** $p<0.0001$.

SL dose-dependently increase ROS expression in HT29 cells

The treatment of CCD-841-CoN cells with ASL caused a significant increase in in the fluorescent intensity at concentrations of 80µg/ml (Fig 5.4a $p < 0.05$), a relatively high concentration compared to the 40µg/ml ASL concentration needed to induce a significant increase in the fluorescent intensity seen in HT29 CRC cells (Fig 5.4b $p<0.05$). The levels of ROS produced by CCD-41-CON cells subjected to ASL was less than the ROS produced by H_2O_2, a control known to elevate ROS expression *in vitro*.

ASL further increased the fluorescent intensity ofHT29 cells ($p<0.0001$) above concentrations of 40µg/ml in a dose dependent manner. However, LSL treatments induced an elevation in the fluorescent intensity observed in CCD-841-CoN cells at 10µg/ml (Fig 5.4c $p < 0.05$) with further increases in the fluorescent intensity observed up to 80µg/ml. This was higher than the amount of ROS expression with the control H_2O_2. In the HT29 cell line, an elevation in the fluorescent intensity was only evident from doses of 60µg/ml LSL (Fig 5.4d $p < 0.05$) upwards. The fluorescence values were corrected for cell number.

Figure 5.4 SL dose dependently induces ROS production in HT29 CRC cells *in vitro*

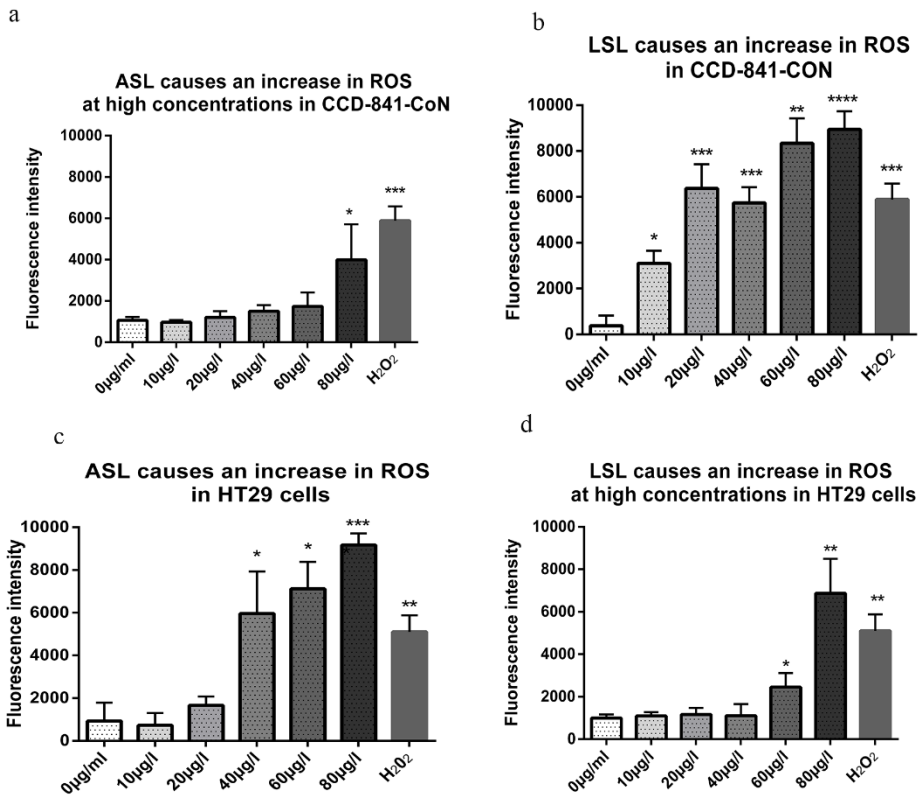


Fig 5.4: Quantification of ROS production caused by 0, 10, 20, 40, 60 or 80µg/ml treatments of ASL and LSL in CCD-841-CoN and HT29 cells via fluorometric analysis. a; A significant increase in ROS production was only observed at 80µg/ml in CCD-841-CoN cells treated with ASL (* $p<0.01$ a). However a significant increase in ROS production was observed from 40µg/ml ASL in HT29 (* $p<0.01$ b) with a dose dependent increase up to 80µg/ml (*** $p<0.0001$). However LSL increased ROS production from 10µg/ml in CCD-841-CoN (c) but only induced significant ROS production after 60µg/ml in the CRC HT29 cell line (* $p<0.01$ d). Graphs representative of mean ± SEM. Data representative of n=3 with three independent replicates. Statistical significance was determines using a students t-test and one-way ANOVA. * $p<0.01$, **$p<0.001$, *** $p<0.0001$.

5.5 Discussion

Local and metastatic recurrence of CRC commonly occurs, even when tumours are completely excised during surgical treatment. Following surgical resection, the current gold standard drug regimen used in the treatment of CRC in the UK, is a cocktail of chemotherapeutic agents made up of folinic acid, fluorouracil and oxalipatin (FOLFOX) (Mohelnikova-Duchonova et al., 2014). This combination of drugs is given by *i.v.* injection and primarily acts on residual CRC cells by blocking DNA replication. Although FOLFOX is the first-line protocol, only 40-50% (Longley et al., 2003) of those prescribed this regimen, respond to the treatment due to intrinsic or acquired drug resistance in the remaining patients (Chen et al., 2013). However, like other chemotherapeutic regimes, FOLFOX does not distinguish between normal or cancerous cells resulting in such side effects as hair loss (Taïeb et al., 2005), a reduced immune response and neurotoxicity. As the current gold standard chemotherapeutics have restricted efficacy and severe side-effects, new approaches for treatment of CRC progression are warranted which target cancer cells whilst proving non-toxic to normal tissue cells. In chapter four, we have shown the ability of ASL to differentiate between normal or colon cancer cells, potentially paving the way to an alternative therapy giving a more natural anti-cancer therapy option any perhaps reducing the risk of resistance.

Although the cytotoxicity of SL mixtures has been widely reported against pancreatic (Fu et al., 2008), lung (Chen et al., 2006b), esophageal (Shao et al., 2012) and breast cancer (Ribeiro et al., 2015) cell lines *in vitro*, the mechanism of how they inflict their toxicity is not clearly understood as only changes in viability was measured. Here we investigated the potential pathway of which purified ASL and LSL inflict a response in the normal colon CCD-841-CoN and CRC cell line HT29, which is commonly used to test the chemotherapeutic potential of compounds *in vitro* (Zweibaum et al., 2011, Baricault et al., 1995).

It has been proposed that the lipid structure of BS allows the molecules to interfere and integrate with the lipid bilayer. One of the major components of the lipid membrane in mammalian cells are the clusters of lipid rafts which play an essential role in cell signalling (Simons and Toomre, 2000). Previous studies have shown that lipid raft disruption by pharmacological agents such as lovastatin aids in inhibiting the growth of MCF7 breast cancer cells *in vitro* (Owens et al., 2013). The HT29 cell line is a robust adenocarcinoma colonic cancer line often used, on its own, in pilot experiments to test the effects of candidate therapeutics against CRC. This cell line was one of the first CRC cell lines isolated and expresses many of the characteristics of mature intestinal cells (Martinez *et al.*, 2015). To date, a majority of studies carried out investigating the *in vitro* effects of drugs on lipid raft disruption in CRC, have been carried out solely on HT29 cells (Simons *et al*., 2011, Lacour *et al.*, 2004). Therefore, in order to compare the results obtained in this chapter against previously published studies, we tested the ability of purified ASL and LSL to disrupt the membrane lipid rafts of HT29 cells.

ASL induced a dose dependent disruption and changes of the lipid raft component of the membrane visible by an increase in fluorescent signal intensity in the HT29 cancer cells. Both ASL and LSL caused alterations to lipid raft expression, when quantified using fluorescent intensity. Previous studies have shown that an increase in fluorescent intensity located in specific regions of the lipid raft, similar to those present after SL treatment, has been associated to receptor clusters in response to a stimulus (Simons and Ikonen, 1997). Similar cluster formations, to those observed in ASL treatments, has also been reported in HT29 cells treated with Cisplatin, an anti-cancer drug which showed that these clusters formed in the HT29 cells treated with Cisplatin stained positive for lipid rafts and also stained positive for the death receptor CD95 (RAS) hypothesising that activation of the lipid raft cluster was caused by the activation of the RAS receptor and resulting in apoptosis of the cell (Lacour et al., 2004) and has also been reported HaCaTs subjected

to ultraviolet B light irradiation (Wu and Wu, 2012). This mechanism could potentially explain the apoptosis and necrosis observed in chapter three and four when cancer cells were treated with SL.

It has been proposed that these lipid clusters are receptor patches within the lipid domains which accumulate and migrate towards a single cellular pole forming a cap. This causes the reorganisation of the cytoskeleton, a process that is highly dependent on energy expenditure and consequently allows alterations to signal transduction in cancer cells (Graziadei et al., 1990).

Lipid rafts play a role in the regulation of tight junctional structure *in vitro* (Laing et al., 2005, Head et al., 2014) which was shown by Lambert and coworkers using cholesterol depleting agents which disrupt the cholesterol-rich domains within the lipid membrane (lipid rafts) resulting in an increase in tight junction permeability by interfering with tight junction protein expression (Lambert et al., 2008). We observed a dose dependent decrease in ZO-1 expression, a key component of tight junction regulation, in HT29 cells treated with SL. ASL demonstrated a superior effect, causing a dose dependent disruption to the tight junction complexes in HT29 via downregulation of ZO-1. LSL treatment resulted in the downregulation of ZO-1 in CCD-841-CoN at relevantly low doses however HT29 cultures at concentrations but failed to have any effect on HT29 until higher doses. Studies carried out in CRC Caco-2 cell *in vitro* have shown that lipid rafts play an important role in stabilising tight junctions via mediation of WNT signalling (Lambert et al., 2008). Change to lipid raft configuration ultimately leads to the loss of tight junction integrity (Owens et al., 2013, Lambert et al., 2008) similar to the results observed in this chapter. Future experiments could delve deeper and look at the expression of Z0-1 at a protein level using western blot analysis. This data would also be strengthened with the addition of more cancer cell lines and the normal cell lines. The

addition of a drug which can inhibit ZO-1 expression such as cisplatin (Baribeau *et al.,* 2014).

In this chapter, experiments were designed to investigate the effects of SL on the MMP and ROS expression. Over the years it has been widely accepted that there is a strong correlation between MMP regulation and ROS production. Many manuscripts have been published to test this theory including studies by Bonora *et al.,* (2012). Mitochondria have been linked to being the main source of ROS in the cell. ROS generated by mitochondrial stress play an important role in the release of cytochrome c and other cellular death proteins which in turn activates the cell death cascade (Ott *et al.,* 2007). ROS are expressed at low concentrations in cells under normal conditions as it plays a role in intracellular signalling however when elevated, ROS is a key inducer of cellular death (Simon et al., 2000). The ability of SL to increase the expression of ROS in culture has been previously reported in MDA-MB-231, an adenocarcinoma cell line, when treated with 12µM of purified LSL (92%) (Ribeiro et al., 2015) which is the equivalent to approximately 140µg/ml of our SL. In this chapter we observed a significant increase in ROS production in HT29 treated with ASL at concentrations starting at 40µg/ml and only affected while elevated ROS expression was observed in CCD-841-CoN cells from 80µg/ml. Alterations to the expression of ROS after SL treatment has also been observed in MCF- breast cancer cell lines treated with 30µM of surfactant - Surfactan (Cao et al., 2010).

The data obtained in this chapter demonstrating that SL administration may influence the expression of MMP and ROS may help to explain why a change to cell viability and increase in cell death that was seen in the *in vitro* components of chapter three and four. As mentioned above, the MMP and ROS relationship has been shown to result in the activation of cell death mechanisms. It has also been reported that mitochondrial induced

ROS regulation can stimulate the production of proinflammatory cytokines especially in malignancies and other diverse pathological conditions (Li *et al.*, 2013). It has also been reported that several transcription factors (TF) present in the nucleus are also present in the mitochondria. These include p53, nuclear factor kappa B, STATs and HIF-1 (Zhang *et al.*, 2007, Handy *et al.*, 2012). These TFs have also been shown to be effected by ROS production also.

The primary data obtained in this chapter opens up a new avenue of prospective future mechanistic studies which could look deeper into the relationship between SL mediated regulation of MMP expression, ROS production and how they can regulate the TFs mentioned above. This could give a better insight on how SL target a cancerous cell and regulate cell death.

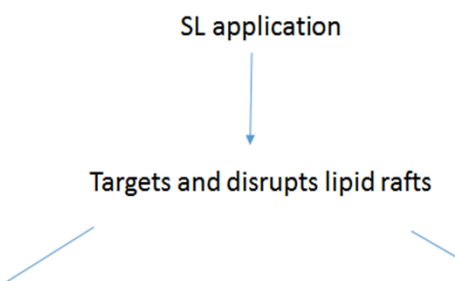
It has been previously documented that lipid-based compounds such as Haloperidol, an antipsychotic drug which works by altering receptor clusters, (Otzen, 2016, Alves et al., 2011) specifically G-coupled receptors present in the lipid raft compartment of the lipid membrane of SH-SY5Y neuroblastoma cells (Sánchez-Wandelmer et al., 2009). We hypothesise that SL work in a similar manner to lipid-based compounds due to their ability to redistribute lipid raft structures inducing raft clusters in the membrane. At low concentrations, SL had the ability to disrupt tight junctional complexes, which play an important role in the regulation of cell adhesion and migration (Tokes et al., 2013). Changes to tight junctions within lipid rafts result in alteration of cell migration (Murai, 2011) and inhibition of the invasive properties of cancer cells (Owens et al., 2013). The disruption of lipid raft formation and structure is known to influence the release of ROS and is linked to the induction of apoptosis and necrosis (Higuchi et al., 1998). Therefore, the results reported in these studies and also seen in this chapter, may explain how, at low concentrations, SL demonstrated the ability to influenced the migration and invasion of

CRC cells and at higher concentrations, mediate apoptotic and necrotic death in CRC as outlined in chapter three and four.

Other anti-cancer drugs incorporating lipid structures such as edelfosine, an alkylphospholipid analog, has been shown to specifically target the cell membrane of cancer cells via lipid raft disruption (Gajate and Mollinedo, 2007) resulting in the inhibition of migration in an non-adherent Jurkat lymphoma cell line as well as the adherent MDA-MB-435 breast cancer cell line (Potier et al., 2011). Similar to the results observed using ASL, edelfosine has the ability to differentiate between normal and cancer cells, by sparing normal cells from toxicity and apoptosis (Gajate and Mollinedo, 2014).

In conclusion, we showed that SL treatment results in lipid raft redistribution *via* cluster formation and caused the downregulation of ZO-1 expression in HT29 CRC cells. This cell membrane disruption may underlie the changes in ROS and MMP expression observed in the CRC cell lines induced to undergo an increased level of apoptotic and necrotic cell death in response to LSL and ASL (Chapter 3 & 4 respectively). However, a deeper mechanistic study would need to be carried out, with the inclusion of the other cell lines used in this thesis, to gain a better insight into how the purified SL regulate the death of cancer cells.

Figure 5.5 Schematic of the potential anticancer mechanistic of SL



Chapter 6

General Discussion

6.1 Discussion

The treatment of CRC is challenged by lack of recommended screening compliance, late diagnosis of sporadic CRC, cytotoxicity of chemotherapy agents, drug resistance and CRC relapse. A method to combat the increasing desire for chemotherapeutic agents with

less cytotoxic side effects has involved the testing of naturally occurring agents derived from plants, marine, aquatic or microbiological origins (Newman and Shapiro, 2008). Since the 1940's, over 60% of new pharmacological agents can be traced back to a natural origin (Demain and Vaishnav, 2011). This has increased to over 74% since 2002 (Cragg and Newman, 2005) and include Taxol (plant derived used to treat ovarian and breast cancer) (Weaver, 2014). A number of naturally derived compounds such as Salinosporamide A (isolated from *Salinispora tropica* for the treatment of multiple myeloma) (Harrison et al., 2016) and Rebeccamycin (isolated from *Streptomyces* sp. for the treatment of lung, breast, liver and kidney cancer) (Eckel and Schmid, 2014) are currently undergoing clinical trial testing in humans (Harvey, 2008).

Due to the success of chemotherapeutics derived from naturally occurring compounds used in the treatment of cancer and metastatic disease, there is an increasing demand for the search of naturally-occurring novel compounds for use in the treatment of CRC.

This aim of this thesis was to test the anti-cancer potential of purified SL congeners by evaluating their ability to reduce CRC cell viability, induce cell death and inhibit the migration and invasion properties of cancer cells *in vitro.* This thesis also aimed to determine a therapeutic window for SL congeners in cancer cell lines without inducing toxicity to normal colonic cells *in vitro.* This thesis also aimed to address the lack of *in vivo* anti-cancer assessment of purified SL by using the $Apc^{min+/-}$ mice to assess the potential benefit of LSL or ASL treatment in a murine model of CRC progression. Finally, this thesis explores the possible mechanisms of for their chemotherapeutic effect on CRC cell lines.

The literature is currently populated with several studies which explore the anti-cancer properties of BS in vitro, including SL, however the range of responses observed may be explained by the differing compositions and purities of the mixtures tested.

The differences observed during our in vitro and in vivo studies for assessing SL activity (as described in Chapter one) identified a number of issues, which we believe to be generic in the current approach to studies on SL using bioassays:

1. Studies are carried out using crude mixtures which are neither purified nor well characterised.
2. Studies are carried out on a variety of different cancer cell lines, that are unrelated: in one study a liver (HepG2), lung (A549) and breast (MCF-7) cancer cell line were examined (Rashad et al., 2014). To assess specificity, is important to include a sufficient number of similar cancer types from the same tissue of origin and their efficacy across different stages (adenocarcinoma vs carcinoma, neoplasm vs metastatic). This was the approach in our studies, where 5 of the cell lines represented the range of CRC pathophysiology (Wilding and Bodmer, 2014).
3. Several manuscripts examining the effects of SL omit normal cell controls and often data sets are not included in the manuscripts. Any normal cell lines that are used are of different origin and non-adherent thus bearing no similarities to the cancer cell lines tested.
4. The anti-cancer mode of action *in vitro* is rarely investigated.
5. Anti-cancer studies are rarely carried into a relevant tumour model for *in vivo* testing of the compound. There is only one other report of an *in vivo* model, where the effects of a crude SL mixture with an unknown composition was examined in a cervical cancer (HeLa) xenograft model (Li et al., 2016).

The use of purified preparations of SL instead of crude mixtures:

To address the issues described above, we use a highly purified preparation of LSL and ASL and tested them on a range of CRC cell lines, which best incorporated the different

stages of the cancer development and coupled it with a normal colonic epithelial cell line and a lung fibroblast cell line. We next administrated the purified LSL and ASL, orally, to the $Apc^{min+/-}$ mouse, a pre-cancerous tumour model frequently used in CRC studies (chapters three and four). Finally, we investigated a potential method of how SL exerts their biological activity against CRC (chapter five).

SL congeners (composed of a hydrophobic fatty acid and a hydrophilic carbohydrate head) occur in varying amounts within crude mixtures and demonstrate differences in both carbon chain length and their degree of acetylation (Rau et al., 2001). Due to time constraints, SL are generally produced as crude mixtures with low quantity retrieval in order to reduce production costs (Mukherjee et al., 2006). For SL to be considered for use as a therapeutic agent, the characterisation and purification of the product is essential. This is to ensure the exact composition of the compound is known and each component is safe for potential clinical use and to ensure the formulation is consistent from batch to batch (Raskin and Ripoll, 2004). It has previously been shown that ASL are the easiest SL congener to obtain and purify as it is the first to be produced in the bioreactor and further purification of the sample can be performed via a hydrolysis reaction of SL (Asmer et al., 1988, Van Bogaert et al., 2007).

New advances in biotechnology have enabled a more cost effective method of purification of LSL. One methodology which was used to generate the batch samples for this thesis involved induction of overexpression of *S. bombicola* lactone esterase (SBLE) allowing the lactonization (esterification) of ASL to form a highly purified LSL product (Roelants et al., 2016). In this thesis, the aforementioned method was used to produce single batches of either diacetylated C18:1 LSL or ASL, which were shown to be 96% (chapter three) and 94% (chapter four) pure respectively.

The potential of SL in biomedical applications have been well established with a diversity of studies claiming anti-microbial (Sleiman et al., 2009), anti-inflammatory (Hardin et al., 2007, Mueller et al., 2006), anti-viral and spermicidal properties (Gross et al., 2004, Shah et al., 2005) *in vitro*, although few show their effectiveness *in vivo* (Bluth et al., 2006, Li et al., 2016).

The ability of crude SL preparations to reduce cell viability and/or induce cell death has been documented in pancreatic (Fu et al., 2008), liver, lung (Chen et al., 2006), oesophageal (Shao et al., 2012), leukemic (Joshi-Navare et al., 2011) and breast (Ribeiro et al., 2015) cancer cell lines *in vitro.* Generally, mixtures with a high percentage of LSL, (specifically C18:1 structures), show the greatest potency when compared with ASL (Ribeiro et al., 2015).

In chapter three, we demonstrate that LSL exert a cytotoxic effect in CRC cell lines, resulting in a reduction of CRC and normal cell viability at similar doses therefore concluding that there is no therapeutic window of which LSL can inflict a toxic effect on the CRC cells without any adverse effects on the normal cell lines. In contrast to other available studies, LSL is reported to show a wide therapeutic range with reductions in viability at concentrations as low as 15µg/ml+ in MDA-MB-231 breast, 30µg/ml+ in H7402 liver, 40µg/ml+ in A549 lung and as high as 500µg/ml and 2mg/ml in HPAC pancreatic cancer cell lines *in vitro* (Ribeiro et al., 2015, Chen et al., 2006, Fu et al., 2008). These studies have also reported the ability of LSL rich mixtures to differentiate between normal and cancer cell lines showing no toxicity to a non-adherent, circulatory monocyte cell line (PCBM) or an uncharacterised cell line (Chang) (Chen et al., 2006) later shown to be contaminated with HeLa cells (Gao et al., 2011).

Our results established the ability of ASL to reduce the cell viability of CRC cells at concentrations of 20µg/ml. Under the same conditions, there were no change to the

viability of the normal control CCD-841-CoN cell lines. This has only been reported in one other study, in a pancreatic cell line HPAC, where viability was reduced with doses of 500µg/ml and complete inhibition of viability observed at 2mg/ml (Fu et al., 2008).

The anti-cancer potential of other BS compounds have also been reported. A preparation of a lipopeptide produced by *B. circulans* with undisclosed purity, reduced the viability of HT29 and HCT15 cell lines by 90% with doses of 300µg/ml in both cell lines after 24h and deemed the IC_{50} values at120µg/ml and 80µg/ml respectively. The study also deemed the IC_{50} of NIH/2T2, a mouse adherent fibroblast cell line, to be 500µg/ml, five times higher than the cancer cells. Interestingly, the study states that crude preparations of the compound had little to no anti-cancer potency which can be explained by the low concentrations of lipopeptide present in the mixture (Sivapathasekaran et al., 2010) while the biosurfactants surfactant isolated from *Bacillus subtilis* reduced the viability of LoVo colorectal cancer cell lines at doses of 50µg/ml to 80% cell death after 24h (Kim et al., 2007).

To date, the majority of SL studies investigate cytotoxicity and viability of cancer cells *in vitro*. An exception to this a report by Chen (Chen et al., 2006) in liver cancer cells (H7402) where changes in cell morphology were observed (cells appearing rounded with condensed chromatin). This study demonstrated that doses of 40µg/ml of SL mixtures resulted in an influx of stationary G1 and S1 phase cells, an increase in intracellular Ca^{2+} and a rise in apoptotic positive cells (Chen et al., 2006). Our results have shown that LSL causes morphological changes which are observed in the normal CCD-841-CoN cell lines with cell rounding and detachment evident. In the CRC cell lines there are signs of cell detachment from 40µg/ml LSL. The detached cells counted in the supernatant tested positive for cell death while 70µg/ml resulted in obvious signs of cell rounding and large areas devoid of cells. ASL treatments resulted in no observable morphological changes

in the normal CCD-841-CoN cells. However, similar to LSL, ASL resulted in morphological changes in the CRC cells at both concentrations. Cell rounding has also been reported in a melanoma cell line (A 375), when treated with pseudofactin, a biosurfactants produced by *Pseudomonas fluorescens* BD5 from 7µM (equivalent to 4.8mg/ml LSL used in this thesis) after 24h. At this concentration, pseudofactin also failed to induce morphological changes in Normal Human Dermal Fibroblast (NHDF) cells and Normal Human Epidermal Keratinocytes (NHEK) cells *in vitro* (Janek et al., 2013).

It is vital that potential chemotherapeutic agents must be able to do to more than just reduce the viability and induce cell death in cancer cells. Although these constitute an important part of cancer development, it is important that anti-cancer agents, such as SL, have the ability to influence the remaining hallmarks of cancer which include metastasis and invasion (Hanahan and Weinberg, 2011).

Metastasis is defined as the movement of cells that originate from primary neoplasms to distal organs and proves to be one feared complication of CRC. Although there has been prodigious development in the diagnosis and treatment of the primary neoplasms in CRC, metastasis is still the major cause of deaths associated with this cancer and is caused by cells resistant to treatment (Fidler, 2003).

Migration is an essential part of the metastasis cascade in cancer and plays a key role in the migration, invasion, intravasation and extravasation process of tumour cells. The measurement of migration and invasion, the first major steps, can be easily measured *in vitro* using the scratch assay or a Boyden chamber (Roussos et al., 2011). The effects of SL on cancer cell migration was assessed in chapters three and four.

The CRC cell lines HT29 and HT115 are derived from patients with adenocarcinoma and aggressive carcinoma tumours respectively and their use has risen in popularity to study

the CRC pathogenesis and the response to chemotherapeutics (Lengauer et al., 1997, Lacour et al., 2004, Coates et al., 2007). We employed these cell lines to investigate the effects of SL against metastatic properties *in vitro* and demonstrated LSL had a differential effect and resulted in the exacerbated the migration of HT29 and HT115 cells across the scratch compared to the vehicle alone, at 10µg/ml, a relatively low dose while at the same dose, LSL completely inhibited the migration of the normal CCD-841-CoN cells. In comparison, the results obtained from chapter four demonstrated the ability of ASL to reduce the migration of HT29 and HT115 compared to the vehicle control, without inhibiting the migration of the normal CCD-841-CoN cells. A previous study evaluating the effects of a 92% C18.1 diacetylated pure LSL preparation against a breast cancer cell line MDA-MB-231, also tested their anti-migratory potential via the starch assay. LSL demonstrated the ability to reduce the migration of MDA-MB-231 cells by 41.5% after 24hr (Ribeiro et al., 2015). It may be concluded the biological effect of SL is dependent on the cancer/tissue type.

Although the scratch assay is a good indicator of migration, the Boyden chamber proves more advantageous as it measures the movement of cells across a porous membrane in a response to a stimulus. The Boyden chamber can also be modified by adding a layer of extracellular matrix (ECM) allowing cancer cells to invade through the ECM before migrating through the porous membrane. In chapter three, LSL resulted in 35% inhibition of migration at doses of 50µg/ml and above while in chapter four, ASL decreased the migration of cancer cells by almost half from only 10µg/ml. It can be concluded that ASL are more efficient at reducing the metastatic properties of CRC cell lines HT29 and HT115 and yet again proved to be non-toxic to the normal CCD-841-CoN colonic cells when compared to LSL. This first time the Boyden chamber has been used to assess the anti-metastatic properties of SL.

This thesis focusses on the evaluation of five colorectal cancer cell lines which differ by type (adenocarcinoma (HT29) vs carcinoma (HCT116)) and stage of cancer such non-metastatic or metastatic. The data presented in the *in vitro* proportion of chapter three and four demonstrates a high potency of SL against CRC cells and strongly suggests that ASL has the ability to differentiate between cell lines inflicting a chemotherapeutic effect in the cancer cell lines alone compared to LSL. This is a highly sought after chemotherapeutic trait which has been a novel concept since being proposed by Blagosklonny and Pardee. They believe that chemotherapeutic drugs should target tumours via a nongenomic altering mechanism and kill cancer cells quickly (days)(Liu et al., 2015) rather than weeks or months which is the case for the chemotherapeutic currently used. This is highly possible as many *in vitro* studies demonstrated the ability of drugs to kill cancer cells with an hour while in chapter three and four, SL have been shown to reduce viability and induce cell death within 24h. This potential fact acting method of hitting tumours with an initial hard dose may reduce the side effects often associated with cancer drug treatments such as immunodeleption and due to these cell types such as bone marrow growing quicker than the tumour cells thus can be replaced with healthy cells after the initial hard dose (Liu et al., 2015).

As aforementioned, the *in vitro* experiments carried out highlight a clear difference between the interactions of LSL and ASL when applied to both cancerous and normal colon cell lines. Although these congeners both belong to the same family known as SL, they both differ in their own ways. LSL is the later product to be retrieved from the batch ferminatator, has a basic pH and consists of a closed ring structure. In contrast, ASL is the first congener to be produced, is more acidic in nature and presents as an open ring structure. These stark differences, especially in relation to the structure have shown to play a role in the biological activity a compound. This phenomenon has also been noted in the testing of statins as anti-cancer agents *in vitro*. It was demonstrated that a closed

ring statin structure, such as mevastain, had a potent effect on a range of cancer cells lines while an open ring structure, such as pravastatin, had little to no effects on the cancer cells.

One of the most significant results obtained from the *in vitro* component of this thesis was the discovery that ASL had the ability to differentiate between normal and cancer cell types. It is still unknown exactly how the ASL congener has the ability to differentiate between cancer and normal cells as seen in chapter four. One possible theory for this could be the ability of ASL to be fast acting killing cells that divide more rapidly – eg cancer cells (in culture: HT29 has a doubling time of 12hrs while normal CCD-841-Con is 36hrs). This theory could be carried out by comparing the time it takes cancer and normal cells to die when treated with various concentrations of LSL and ASL *in vitro.* Other drugs such as cyclophosphamide are well known to work by killing fast dividing cancer cells (Chan et al., 2012) such as leukaemia and small cell lung carcinoma (Reeder *et al*.,2009) .

Due to lack of *in vivo* evaluations investigating the inhibitory influence of SL in an *in vivo* model of cancer we focused on the effects of purified LSL (chapter three) and ASL (chapter four) in a pre-cancerous model of CRC. We hypothesised that that oral administration of SL inhibited the development of neoplasms in the $Apc^{min+/-}$ mouse model. Mice were fed orally to allow direct access of the SL to the intestinal and colonic epithelial to exert a therapeutic effect.

The treatment of $Apc^{min+/-}$ mice dosed with 50mg/kg LSL resulted in an increase in the number and size of polyps found in the small intestine. These mice have a predisposition to splenomegaly and reduced haematocrit, resulting in anaemia (You et al., 2006). LSL treatments resulted in a further increase in spleen size and a reduction in haematocrit levels. This could further decrease in the lifespan of the $Apc^{min+/-}$ mice (Holcomb et al.,

2008). In contrast, although ASL did not cause a change in tumour number or size, there was a significant decrease in the spleen size and haematocrit levels. This proves a highly significant result as these mice tend to die as a consequence of the anaemia rather than neoplasm growth (Holcomb et al., 2008). The development of anaemia has been noted as a serious issue in FAP patients also (DeCosse et al., 1989) which is caused by gastrointestinal bleeding caused by epthelial eroision caused by polyp development (Adolph and Bernabe, 2008). A study carried out by (Hasty et al., 2014) demonstrated an increase in life span of $Apc^{min+/-}$ mice treated IV with eRAPA, an anti-aging drug. They also correlate the increase in haematocrit levels (vehicle 17% vs 45% eRAPA) as a result of reduced blood loss, as a contributing factor to the increased life span seen after treatment (Hasty et al., 2014).Although the ability of ASL to help project the haematocrit and spleen can be seen as promising data, it would be further strengthened by the addition of a well-known chemotherapeutic agent such as cisplatin to compare against the efficacy of SL.

The *in vivo* component of this thesis clearly investigates the biological activity of LSL and ASL in animal model of FAP. As mentioned previously, this is the most widely used model to investigate familial CRC syndromes. However, this model does not fully encapsulate all aspects of CRC developed as seen in FAP patients as it only produces benign tumours along the intestinal tract while humans develop both benign and malignant tumours in the colon. This is a major limitation of the study and in order to truly investigate the appropriateness of SL as a potential anti-cancer agent, an extra study would need to be carried out testing the biological activity in a malignant colon model in vivo. This will be discussed in more detail in the future experiments section of this thesis.

After the publication of the first manuscript derived from the results of this thesis [Chapter 3] (Callaghan et al., 2016) an article by Li *et al,* (Li et al., 2016) reported on the ability of

a crude SL preparation to reduce the tumour burden in a HeLa xenograft model using BALB/c nude mice and demonstrated a dose dependent decrease in tumour burden. This study, however, warrants caution for interpretation of experimental results as this amount of ethanol could result in long-term side-effects especially where dosing exceeds the 12-day treatment period used in this paper. Consistent with this, another study using 80% ethanol administered intragastrically for 28 days was used to induce alcoholic liver in an animal model (Ji and Kaplowitz, 2003).

Chapter 5 explores the possible mechanisms by which SL exert their biological actions. We hypothesised that SL disrupt membrane organisation via lipid raft integration within CRC cells resulting in a dose dependent downstream effect on cell adhesion and induction of cell death (as seen in chapters three and four) tight junction disruption, influence MMP and ROS production (chapter 5).

It has been hypothesised that SL cause cell death via their strongly amphiphilic properties, which cause physical changes to the lipid bilayer. A study by Isoda et al, used SL mixture supplementation in a glioma cell line (HL60), which resulted in a decreased cell viability and inhibited protein kinase C activity (PKC) which plays a vital role in the modulation of membrane structure, transcription and cell proliferation regulation (Nishizuka, 1984). It has been reported that glycolipids isolated from *Gigartina tenella* induce changes to the lipid raft structures by inducing the aggregation of rafts at the membrane surface, a phenomenon which is microscopically visible via fluorescent dyes and is correlated to activation of receptors such as death receptor (FAS)s (Zhang et al., 2006). This clustering of raftsresulted in the disruption to the actin cytoskeleton structure in the JURKAT leukaemia cell line (Vali et al., 2005). In comparison to the primary cell cultures or non-transformed cells, it has been hypothesised that cancer cell lines have varying levels of lipid rafts with different structures, compared to normal cells, thus rendering then unstable

(Patra, 2008). We have shown that both ASL and LSL have the ability to alter raft formation by increasing cluster formations on the surface of the cell membrane. This cluster formation have been shown to be caused by receptor activation (Simons and Toomre, 2000) in response to a stimulus such as chemotherapeutics and can result in disruption to raft integrity (Gajate et al., 2009). Lipid raft integrity is essential for maintain cell homeostasis because of their fundamental use as signalling platforms for the activation of receptors, kinases (George and Wu, 2012) and calcium channels (Berthier et al., 2004) , which are all key players in the mediation of cell death in cells. This makes lipid rafts a potential anti-cancer drug target (Li et al., 2006).

It has been shown that changes to lipid raft structure via receptor activation plays an important role in the disruption of cell adhesion via tight junction deregulation. An important player in this process is ZO-1, which is a member of the occludin protein family. ZO-1 is a key role as a scaffold protein which holds together fibril structures in the lipid bilayer to the cytoskeleton thus affecting cell adhesion (Itoh et al., 1997). As it is well known that BS can disrupt the adhesion of cells, we investigated if SL disrupts ZO-1 in cells which may give an insight into their mechanism of action.

We demonstrated the ability of ASL to influence ZO-1 expression at 10µg/ml ASL. Studies have shown that alterations to ZO-1 can induce changes in cell migration and disrupt CD44 expression in cells resulting in the reduction of adherence (Okada et al., 1996, Harley et al., 2008) which may explain why we observed a reduction in cell migration in response to ASL treatment in chapter four.

The increase of ROS production promotes cell death in cells (Wang and Yi, 2008). In chapter five, we also demonstrated the ability of SL to increase ROS production in HT29 cells. The ability of SL to increase ROS production has also been shown in the breast cancer cell lines MDA-MB-231 when treated with 120µg/ml and 150µg/ml of C18:1

diacetylated LSL (Ribeiro et al., 2015). Similar results have been shown in other BS such as surfactan, which has been shown to induce cell death via elevation of ROS production in MCF-7 cells (Cao et al., 2010) *in vitro*.

The increase of ROS production has also been correlated to the disruption of lipid rafts found in the cell membrane caused by pathological modification such as disease development (Alzheimer's or Atherosclerosis (Ehehalt et al., 2003)) or drug administration (Edelfosine, a chemotherapeutic) which can result in the signalling of cell death (Gniadecki et al., 2002) (Circu and Aw, 2010) by apoptosis and/or necrosis (Fiers et al., 1999), which is similar to the results we observed in chapter three and four.In addition to testing the anti-cancer properties SL, we also tested the bioactivity of Bolaform SL (BfSL) (Appendix 1.2) and glucolipids (Appendix 1.3). These differing classes of SL are produced in large quantities by strains of *Starmerella bombicola* deficient in acetyltransferase and lactonesterase enzymes. BfSL consists of two sophorose heads attached to either end of a lipid and often contain a small percentage of ASL. BfSL are naturally easier to solubilise, compared to other classes of surfactants, due to the carbohydrate head on each side of the fatty acid (Van Bogaert et al., 2016). BfSL mixtures may prove useful in the purification and transfection of DNA due to their ability to bind and compact DNA (Sohrabi et al., 2013), however any other biological activities are unknown. Glucolipids are a derivative of SL which contain a glucose head replacing the sophorose head. This SL derivative has been first isolated by our collaborators at the Biobase plant in Belgium, therefore any biomedical applications are unexplored.

Our results showed that both bolalipids and glucolipids resulted in a differential effect depending on concentration. Results show a non-linear fluctuation to viability across all CRC cell lines tested. This may be explained due to reduced purity in the samples we obtained. BfSL was 84% pure while the glucolipids 77%. This is a large difference

compared to the 96% and 94% LSL and ASL used in the previous studies. Any leftover reagents from the production process can prove detrimental and will contribute significantly to the bioactivity differences in the compounds obtained from different production batches.

6.2 Conclusion:

The overall aim of this thesis was to characterise the molecular congeners and standardize the in vitro and in vivo models of CRC used to assess biological activity of several different classes of SL produced by the commercially exploited yeast *Starmerella bombicola*. In addition, a partial characterization of the cellular /molecular mechanism of action of these SL was performed on selected CRC lines *in vitro*. Specifically, in this thesis it is shown that:

1. It is possible to obtain a highly purified preparation of SL for preclinical testing.

2. This is the first report of the use of a purified ASL preparation in cancer related studies.
3. SL congeners have a cytotoxic effect against 5 different well characterized CRC cell lines *in vitro.*
4. Dose-dependent administration of ASL reveals differential effects on cell viability in normal *versus* cancer cells *in vitro.*
5. LSL and ASL induce significant levels of apoptosis and necrosis *in vitro*.
6. LSL exacerbates tumour growth, increases splenomegaly and decreases haematocrit levels in the $Apc^{min+/-}$ model of neoplasia.
7. ASL has no influence on tumour number or size in the $Apc^{min+/-}$ model of neoplasia, however a systematic therapeutic effect was observed which reduced splenomegaly and increased haematocrit.
8. SL increases lipid raft expression in CRC cells, a potential molecular-cell based mechanistic explanation.
9. LSL increases the lipid raft expression in HT29 CRCS at a higher dose than in normal CCD-841-CoN. ASL results in an increase in lipid rafts in CRC cells but not in non-transformed "normal" cells.

6.3 Clinical significance:

Overall the data collected for this thesis shows that purified ASL preparations have the potential to treat people diagnosed with FAP in the clinic. Although ASL administration demonstrated no ability to reduce the polyp burden, it did however, improve the haematocrit levels of the mice and reduce the observed splenomegaly. This could have a large impact on people living with FAP as an enlarged spleen and anaemia can result in abdominal pain, severe fatigue, increased chance of infection and can also reduce the quality of life. Currently there are no agents on the market that can help regulate these

secondary complications therefore the production of an ASL drug to target this would have a major impact in the clinic by improving the patients' ability to live life and reduce the amount of hospital visits and stays.

6.4 Study limitations:

Although this thesis demonstrates a strong correlation between the ability of ASL to regulate the secondary effects associated with FAP *in vivo*, the study its self does not come without its limitations.

The main selling point of the data collected, both *in vitro* and *in vivo*, is in the use of a purified SL compound. A single batch of both ASL and LSL was used throughout the study which was carried out over three years. The purity of the compound was only tested on a single occasion – after the production stage. To improve confidence of the compound purity, MS analysis could have been performed on a regular basis.

The $Apc^{min+/-}$ mouse model is an excellent model for testing drugs. As mentioned in the introduction, it is not a model of CRC and drugs tested in this model cannot be labelled as an anti-cancer agent. This issue can be addressed with more experiments and will be discussed in future experiments section

6.5 Future experiments:

The data collected for this thesis points towards the use of ASL as a candidate drug in the treatments of FAP. However, a plethora of future experiments could need to be carried out in order to fully understand how SL work. These include:

Mechanistic analysis:

An in-depth characterisation of the molecular pathways involved in ASL regulation of CRC cells compared to normal cells. This can be carried out using genomic and proteomic

studies. Not only will this give a deeper understanding into how SL work but it may also give a better understanding on how ASL can differentiate between cancer and normal cells and LSL doesn't.

Synergistic studies:

In addition, further studies could investigate the synergistic effects of SL in combination with current chemotherapeutic treatments for CRC such as 5 fluorouracil. Although the synergic effect of SL with current chemotherapeutics this has never been tested before, it is not a new concept. There have been a number of studies have been carried out on mutant p53 cancer types such as small cell lung carcinoma, testing the synergic effects of current chemotherapeutics with other compounds e.g. cisplatin, an anti-cancer drug used to treat a wide range of cancers coupled with a chemical compound PRIMA-1^{MET}. This combination of drugs reduced the amount of cisplatin needed. A reduction in the use of systemic chemotherapeutics has the potential to reduce side effects associated with their clinical use, such as hair loss, weight loss, sickness and a reduction in immune responsivity (Bonavida et al., 1999).

SL combination studies:

It would be interesting to see the biological effects of different ratios of purified ASL and LSL in combination against polyp growth. While our studies have shown the ASL provided a preferred biological effect compared to LSL, although LSL resulted in an increase in tumour number, it had visible effects on tumour growth while ASL had no effects on the growing polyps. It could be hypothesised that a small percentage (10 – 20%) of LSL with the remaining being ASL may help target and reduce polyp number *in vivo.*

Alternative dosing routes, schedules and concentration *in vivo*:

In this study, mice were administered SL orally, once a day. The most convenient and popular mode of drug administration is oral ingestion as it has a higher patient compliance compared to injection and formulations are cheaper to produce (Krishnaiah, 2010). However one of the major issues with oral ingestion is solubility of compounds that are not readily soluble in aqueous solutions. Oral administration has been linked to slow drug absorption, reduced bioavailability and toxicity of the gastrointestinal tract (Vemula et al., 2010). The ability of ASL to dissolve easily in water is a desirable trait (Savjani et al., 2012) compared to LSL which can only be dissolved in DMSO thus questioning its bioavailability and potential absorption which administrated orally. Other possible routes of administration which could be tested in future experiments include intravenous injections. This method provides good absorption with the drug reaching circulation immediately, provides dependable and has shown to provide consistent reproducible effects, however it needs a highly trained person such as a health care professional to administer it in the clinic, proves more expensive and has a higher risk of toxicity. In vivo testing of IV administered compounds to rodents can cause damage to the tail over time thus limiting the number of dosing occasions.

In this study $Apc^{min+/-}$ mice were dosed with 50mg/kg once daily. Future studies could also look at dosing twice daily or increasing the concentration to 100mg/kg. Changes to the concentration and frequency would first need to be assessed using a pharmacokinetic study to analyse the drug metabolism, bioavailability and elimination in an organism. This can be carried out by dosing a mouse with SL orally, *i.v.* or *i.p,* and withdrawing blood at pre-determined time points (0, 5min, 15mins, 30mins, 1hr up to a 48hr timepoint.) Blood, plasma or serum would be obtained and ran through a mass spectrometer to determine the quantity of drug in circulation. This will help determine a dose and appropriate dosing schedule that will reduce the chances of toxicity and side effects.

This thesis looks at the effects of SL against CRC cells in vitro however in vivo we test their effects against a model of FAP. Although, some would argue that the $Apc^{min+/-}$ model is a model of CRC, it is in fact a benign pre-cancerous model and lacks many of the clinical characteristics of colon cancer. This is a major limitation to this thesis. In order to be able to label ASL as an anti-cancer agent future experiments would need to test SL in an CRC animal model. CRC animal models are severely limited and rarely used. Two models that can be used are:

DMH/AOM model:

The injection of 2-dimethylhydrazine(DMH) or azoxymethane (AOM) into Balb/c mice (Nyce et al., 1984) has proved a popular alternative choice for inducing colon carcinogenesis in rodents (Rosenberg *et al.*, 2008). The injection of these inducer CRC drugs have shown to cause moderate to severe dysplasia along with Unfortunatly time and cost is a limiting factor with this model – induction can take anywhere from six to twelve months. This increases the cost of animal husbandry and coupled with the cost of the AOM and DMH (Juca *et al*., 2014) can prove promblematic for a small budget research centre.

Tumour xenograph model:

An alternative CRC model involves the subcutaneous implantation of HT29 or other CRC cancer cells on to the flank of an immunocompromised mouse (Britten et al., 1999). This is a popular model for directly measuring the effects of a drug on a tumour. This is because the tumour is grown on the back of a mouse and changes to growth can be easily seen and quantified. Although a very popular tool in human drug discover – it has one large limitation – the human cell line is grown in a different host and is not influenced by the normal colonic environment (Richmond *et al.*, 2008).

Chapter 7 References

Adolph, V. R. & Bernabe, K. 2008. Polyps In Children. Clinics In Colon And Rectal Surgery, 21**,** 280-285.

Albini, A., Iwamoto, Y., Kleinman, H., Martin, G., Aaronson, S., Kozlowski, J. & Mcewan, R. 1987. A Rapid In Vitro Assay For Quantitating The Invasive Potential Of Tumor Cells. Cancer Research, 47**,** 3239-3245.

Allen, J. A., Halverson-Tamboli, R. A. & Rasenick, M. M. 2007. Lipid Raft Microdomains And Neurotransmitter Signalling. Nature Reviews Neuroscience, 8**,** 128-140.

Altman, S. A., Randers, L. & Rao, G. 1993. Comparison Of Trypan Blue Dye Exclusion And Fluorometric Assays For Mammalian Cell Viability Determinations. Biotechnology Progress, 9**,** 671-674.

Alves, I., Staneva, G., Tessier, C., Salgado, G. F. & Nuss, P. 2011. The Interaction Of Antipsychotic Drugs With Lipids And Subsequent Lipid Reorganization Investigated Using Biophysical Methods. Biochimica Et Biophysica Acta (Bba)-Biomembranes, 1808**,** 2009-2018.

Amaral, P. F., Coelho, M. A. Z., Marrucho, I. M. & Coutinho, J. A. 2010. Biosurfactants From Yeasts: Characteristics, Production And Application. Biosurfactants. Springer.

Amos-Landgraf, J. M., Kwong, L. N., Kendziorski, C. M., Reichelderfer, M., Torrealba,

J.,Weichert, J., Haag, J. D., Chen, K.-S., Waller, J. L. & Gould, M. N. 2007. A Target Selected Apc-Mutant Rat Kindred Enhances The Modeling Of Familial Human Colon Cancer. Proceedings Of The National Academy Of Sciences, 104**,** 4036-4041.

Asmer, H.-J., Lang, S., Wagner, F. & Wray, V. 1988. Microbial Production, Structure Elucidation And Bioconversion Of Sophorose Lipids. Journal Of The American Oil Chemists' Society, 65**,** 1460-1466.

Ashby, R., Solaiman, D. & Zerkowski, J. 2009. Production And Modification Of Sophorolipids From Agricultural Feedstocks. Biobased Surfactants And Detergents: Synthesis, Properties, And Applications**,** 29-49

Avilés, A., Arévila, N., Díaz Maqueo, J. C. & Nambo, M. J. 1993. Late Cardiac Toxicity Of Doxorubicin, Epirubicin, And Mitoxantrone Therapy For Hodgkin's Disease In Adults. Leukemia & Lymphoma, 11**,** 275-279.

Azim, A., Shah, V., Doncel, G. F., Peterson, N., Gao, W. & Gross, R. 2006. Amino Acid Conjugated Sophorolipids: A New Family Of Biologically Active Functionalized Glycolipids. Bioconjugate Chemistry, 17**,** 1523-1529.

Baccile, N., Babonneau, F., Jestin, J., Pehau-Arnaudet, G. & Van Bogaert, I. 2012. Unusual, Ph-Induced, Self-Assembly Of Sophorolipid Biosurfactants. Acs Nano, 6**,** 4763-4776.

Baccile, N., Cuvier, A. S., Valotteau, C. & Van Bogaert, I. N. 2013. Practical Methods To Reduce Impurities For Gram-Scale Amounts Of Acidic Sophorolipid Biosurfactants. European Journal Of Lipid Science And Technology, 115**,** 1404-1412.

Baccile, N., Babonneau, F., Jestin, J., Pehau-Arnaudet, G. & Van Bogaert, I. 2012. Unusual, Ph-Induced, Self-Assembly Of Sophorolipid Biosurfactants. Acs Nano, 6**,** 4763-4776.

Baccile, N., Cuvier, A.-S., Prévost, S., Stevens, C. V., Delbeke, E., Berton, J., Soetaert, W., Van Bogaert, I. N. & Roelants, S. 2016. Self-Assembly Mechanism Of Ph-Responsive Glycolipids: Micelles, Fibers, Vesicles, And Bilayers. Langmuir, 32**,** 10881-10894

Baltgalvis, K. A., Berger, F. G., Peña, M. M. O., Davis, J. M. & Carson, J. A. 2009. The Interaction Of A High-Fat Diet And Regular Moderate Intensity Exercise On Intestinal Polyp Development In Apcmin/+ Mice. Cancer Prevention Research, 2**,** 641-649.

Bang, B., Gniadecki, R. & Gajkowska, B. 2005. Disruption Of Lipid Rafts Causes Apoptotic Cell Death In Hacat Keratinocytes. Experimental Dermatology, 14**,** 266-272.

Bara, J., Forgue-Lafitte, M.-E., Maurin, N., Fléjou, J.-F. & Zimber, A. 2003. Abnormal Expression Of Gastric Mucin In Human And Rat Aberrant Crypt Foci During Colon Carcinogenesis. Tumor Biology, 24**,** 109-115.

Baricault, L., Denariaz, G., Houri, J.-J., Bouley, C., Sapin, C. & Trugnan, G. 1995. Use Of Ht-29, A Cultured Human Colon Cancer Cell Line, To Study The Effect Of Fermented Milks On Colon Cancer Cell Growth And Differentiation. Carcinogenesis,

16, 245-252.

Barry, M. A., Reynolds, J. E. & Eastman, A. 1993. Etoposide-Induced Apoptosis In Human Hl-60 Cells Is Associated With Intracellular Acidification. Cancer Research, 53, 2349-2357.

Barzilai, A. & Yamamoto, K.-I. 2004. Dna Damage Responses To Oxidative Stress. Dna Repair, 3, 1109-1115.

Berthier, A., Lemaire-Ewing, S., Prunet, C., Monier, S., Athias, A., Bessede, G., De Barros, J. P., Laubriet, A., Gambert, P. & Lizard, G. 2004. Involvement Of A Calcium-Dependent Dephosphorylation Of Bad Associated With The Localization Of Trpc-1 Within Lipid Rafts In 7-Ketocholesterol-Induced Thp-1 Cell Apoptosis. Cell Death & Differentiation, 11, 897-905.

Beutler, J. A. 2009. Natural Products As A Foundation For Drug Discovery. Current Protocols In Pharmacology, 9.11. 1-9.11. 21.

Bisgaard, M. L., Fenger, K., Bülow, S., Niebuhr, E. & Mohr, J. 1994. Familial Adenomatous Polyposis (Fap): Frequency, Penetrance, And Mutation Rate. Human Mutation, 3, 121-125.

Bisht, K. S., Gross, R. A. & Kaplan, D. L. 1999. Enzyme-Mediated Regioselective Acylations Of Sophorolipids. The Journal Of Organic Chemistry, 64, 780-789.

Bissahoyo, A., Pearsall, R. S., Hanlon, K., Amann, V., Hicks, D., Godfrey, V. L. & Threadgill, D. W. 2005. Azoxymethane Is A Genetic Background-Dependent Colorectal Tumor Initiator And Promoter In Mice: Effects Of Dose, Route, And Diet. Toxicological Sciences, 88, 340345.

Bluth, M., Fu, S., Fu, A., Stanek, A., Smith-Norowitz, T., Wallner, S., Gross, R., Nowakowski, M. & Zenilman, M. 2008. Sophorolipids Decrease Asthma Severity And Ova-Specific Ige Production In A Mouse Asthma Model. Journal Of Allergy And Clinical Immunology, 121, S2

Bluth, M. H., Kandil, E., Mueller, C. M., Shah, V., Lin, Y.-Y., Zhang, H., Dresner, L., Lempert, L., Nowakowski, M. & Gross, R. 2006. Sophorolipids Block Lethal Effects Of Septic Shock In Rats In A Cecal Ligation And Puncture Model Of Experimental Sepsis. Critical Care Medicine, 34.

Bonavida, B., Ng, C., Jazirehi, A., Schiller, G. & Mizutani, Y. 1999. Selectivity Of Trail-Mediated Apoptosis Of Cancer Cells And Synergy With Drugs: The Trail To Non-Toxic Cancer Therapeutics (Review). International Journal Of Oncology, 15, 793-802.

Bognolo, G. 1999. Biosurfactants As Emulsifying Agents For Hydrocarbons. Colloids And Surfaces A: Physicochemical And Engineering Aspects, 152, 41-52.

Boileau, T. W.-M., Liao, Z., Kim, S., Lemeshow, S., Erdman Jr, J. W. & Clinton, S. K. 2003. Prostate Carcinogenesis In N-Methyl-N-Nitrosourea (Nmu)-Testosterone-Treated Rats Fed Tomato Powder, Lycopene, Or Energy-Restricted Diets. Journal Of The National Cancer Institute, 95, 1578-1586.

Bonnington, S. N. & Rutter, M. D. 2016. Surveillance Of Colonic Polyps: Are We Getting It Right? World Journal Of Gastroenterology, 22**,** 1925.

Borsanyiova, M., Patil, A., Mukherji, R., Prabhune, A. & Bopegamage, S. 2016. Biological Activity Of Sophorolipids And Their Possible Use As Antiviral Agents. Folia Microbiologica, 61**,** 85-89.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S. & Ouwehand, A. 2012. Food Fermentations: Microorganisms With Technological Beneficial Use. International Journal Of Food Microbiology, 154**,** 87-97.

Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J. & Brattain, D. E. 1981. Heterogeneity Of Malignant Cells From A Human Colonic Carcinoma. Cancer Research, 41**,** 1751-1756.

Britten, C. D., Hilsenbeck, S. G., Eckhardt, S. G., Marty, J., Mangold, G., Macdonald, J. R., Rowinsky, E. K., Von Hoff, D. D. & Weitman, S. 1999. Enhanced Antitumor Activity Of 6-Hydroxymethylacylfulvene In Combination With Irinotecan And 5-Fluorouracil In The Ht29 Human Colon Tumor Xenograft Model. Cancer Research, 59**,** 1049-1053.

Brown, D. & London, E. 1998. Functions Of Lipid Rafts In Biological Membranes. Annual Review Of Cell And Developmental Biology, 14**,** 111-136.

Burish, T. G. 1988. Etiology And Treatment Of The Psychological Side Effects Associated With Cancer Chemotherapy: A Critical Review And Discussion. Psychological Bulletin, 104.

Callaghan, B., Lydon, H., Roelants, S. L., Van Bogaert, I. N., Marchant, R., Banat, I. M. & Mitchell, C. A. 2016. Lactonic Sophorolipids Increase Tumor Burden In Apc Min+/- Mice. Plos One, 11**,** E0156845.

Cameotra, S. S. & Makkar, R. S. 2004. Recent Applications Of Biosurfactants As Biological And Immunological Molecules. Current Opinion In Microbiology, 7**,** 262-266.

Cao, X.-H., Wang, A.-H., Wang, C.-L., Mao, D.-Z., Lu, M.-F., Cui, Y.-Q. & Jiao, R.-Z. 2010. Surfactin Induces Apoptosis In Human Breast Cancer Mcf-7 Cells Through A Ros/Jnk-Mediated Mitochondrial/Caspase Pathway. Chemico-Biological Interactions, 183**,** 357-362.

Casas, J. & García-Ochoa, F. 1999. Sophorolipid Production By Candida Bombicola: Medium Composition And Culture Methods. Journal Of Bioscience And Bioengineering, 88**,** 488-494.

Casteleyn, C., Rekecki, A., Van Der Aa, A., Simoens, P. & Van Den Broeck, W. 2010. Surface Area Assessment Of The Murine Intestinal Tract As A Prerequisite For Oral Dose Translation From Mouse To Man. Laboratory Animals, 44**,** 176-183.

Cavalero, D. A. & Cooper, D. G. 2003. The Effect Of Medium Composition

On The Structure And Physical State Of Sophorolipids Produced By Candida Bombicola Atcc 22214. Journal Of Biotechnology, 103**,** 31-41.

Cayrefourcq, L., Mazard, T., Joosse, S., Solassol, J., Ramos, J., Assenat, E., Schumacher, U., Costes, V., Maudelonde, T. & Pantel, K. 2015. Establishment And Characterization Of A Cell Line From Human Circulating Colon Cancer Cells. Cancer Research, 75**,** 892-901.

Center, M. M., Jemal, A. & Ward, E. 2009. International Trends In Colorectal Cancer Incidence Rates. Cancer Epidemiology Biomarkers & Prevention, 18**,** 1688-1694.

Cetta, F., Dhamo, A., Malagnino, G. & Barellini, L. 2007. Germ-Line And Somatic Mutations Of The Apc Gene And/Or ß Catenin Gene In The Occurrence Of Fap Associated Thyroid Carcinoma. World Journal Of Surgery, 31**,** 1368-1369.

Cesta, M. F. 2006. Normal Structure, Function, And Histology Of The Spleen. Toxicologic Pathology, 34**,** 455-465.

Chan, K., Koh, C. G. & Li, H. 2012. Mitosis-Targeted Anti-Cancer Therapies: Where They Stand. Cell Death & Disease, 3**,** E411.

Chander, S., Lansdown, A., Luqmani, Y., Gomm, J., Coope, R., Gould, N. & Coombes, R. 1994. Effectiveness Of Combined Limonene And 4-Hydroxyandrostenedione In The Treatment Of Nmu-Induced Rat Mammary Tumours. British Journal Of Cancer, 69**,** 879.

Chen, H.-C. 2005. Boyden Chamber Assay. Cell Migration: Developmental Methods And Protocols**,** 15-22.

Chen, J., Song, X., Zhang, H., Qu, Y.-B. & Miao, J.-Y. 2006. Sophorolipid Produced From The New Yeast Strain Wickerhamiella Domercqiae Induces Apoptosis In H7402 Human Liver Cancer Cells. Applied Microbiology And Biotechnology, 72**,** 52-59.

Chen, J., Song, X., Zhang, H. & Qu, Y. 2006b. Production, Structure Elucidation And Anticancer Properties Of Sophorolipid From Wickerhamiella Domercqiae. Enzyme And Microbial Technology, 39**,** 501-506.

Chen, Q., Xia, H.-W., Ge, X.-J., Zhang, Y.-C., Tang, Q.-L. & Bi, F. 2013. Serum Mir-19a Predicts Resistance To Folfox Chemotherapy In Advanced Colorectal Cancer Cases. Asian Pacific Journal Of Cancer Prevention, 14**,** 7421-7426

Chong, E. S. L. 2014. A Potential Role Of Probiotics In Colorectal Cancer Prevention: Review Of Possible Mechanisms Of Action. World Journal Of Microbiology And Biotechnology, 30**,** 351-374.

Chong, K. M., Leelatian, N., Deguire, S. M., Brockman, A. A., Earl, D., Ihrie, R. A., Irish, J. M., Bachmann, B. O. & Sulikowski, G. A. 2016. The Use Of Fluorescently-Tagged Apoptolidins In Cellular Uptake And Response Studies. The Journal Of Antibiotics, 69**,** 327-330.

Christova, N., Tuleva, B., Kril, A., Georgieva, M., Konstantinov, S., Terziyski, I.,

Nikolova, B. & Stoineva, I. 2013. Chemical Structure And In Vitro Antitumor Activity Of Rhamnolipids From Pseudomonas Aeruginosa Bn10. Applied Biochemistry And Biotechnology, 170**,** 676-689.

Church, E., Mehlhaff, C. & Patnaik, A. 1987. Colorectal Adenocarcinoma In Dogs: 78 Cases (1973-1984). Journal Of The American Veterinary Medical Association, 191**,** 727-730.

Circu, M. L. & Aw, T. Y. 2010. Reactive Oxygen Species, Cellular Redox Systems, And Apoptosis. Free Radical Biology And Medicine, 48**,** 749-762.

Coates, E. M., Popa, G., Gill, C. I., Mccann, M. J., Mcdougall, G. J., Stewart, D. & Rowland, I. 2007. Colon-Available Raspberry Polyphenols Exhibit Anti-Cancer Effects On In Vitro Models Of Colon Cancer. Journal Of Carcinogenesis, 6**,** 4.

Corpet, D. E. & Pierre, F. 2003. Point: From Animal Models To Prevention Of Colon Cancer. Systematic Review Of Chemoprevention In Min Mice And Choice Of The Model System. Cancer Epidemiology And Prevention Biomarkers, 12**,** 391-400.

Cox, T. F., Crawford, R. J., Gregory, L. G., Hosking, S. L. & Kotsakis, P. 2013. Mild To The Skin, Foaming Detergent Composition. Google Patents.

Cohen, I. S., Mosher, M. B., O'keefe, E. J., Klaus, S. N. & De Conti, R. C. 1973. Cutaneous Toxicity Of Bleomycin Therapy. Archives Of Dermatology, 107**,** 553-555.

Cohen, J. 2002. The Immunopathogenesis Of Sepsis. Nature, 420**,** 885-891.

Connell, W., Talbot, I., Harpaz, N., Britto, N., Wilkinson, K., Kamm, M. & Lennard-Jones, J. 1994. Clinicopathological Characteristics Of Colorectal Carcinoma Complicating Ulcerative Colitis. Gut, 35**,** 1419-1423.

Cragg, G. M. & Newman, D. J. 2005. Plants As A Source Of Anti-Cancer Agents. Journal Of Ethnopharmacology, 100**,** 72-79.

Crosby, W. H. 1959. Normal Functions Of The Spleen Relative To Red Blood Cells: A Review. Blood, 14**,** 399-408.

Daniel, H.-J., Reuss, M. & Syldatk, C. 1998. Production Of Sophorolipids In High Concentration From Deproteinized Whey And Rapeseed Oil In A Two Stage Fed Batch Process Using Candida Bombicola Atcc 22214 And Cryptococcus Curvatus Atcc 20509. Biotechnology Letters, 20**,** 1153-1156.

Daverey, A. & Pakshirajan, K. 2009. Production Of Sophorolipids By The Yeast Candida Bombicola Using Simple And Low Cost Fermentative Media. Food Research International, 42**,** 499-504.

Decosse, J. J., Miller, H. H. & Lesser, M. L. 1989. Effect Of Wheat Fiber And Vitamins C And E On Rectal Polyps In Patients With Familial Adenomatous Polyposis. Journal Of The National Cancer Institute, 81**,** 1290-1297.

De Gramont, A. D., Figer, A., Seymour, M., Homerin, M., Hmissi, A., Cassidy, J., Boni, C., Cortes-Funes, H., Cervantes, A. & Freyer, G. 2000. Leucovorin And

Fluorouracil With Or Without Oxaliplatin As First-Line Treatment In Advanced Colorectal Cancer. Journal Of Clinical Oncology, 18**,** 2938-2947.

De La Chapelle, A. 2004. Genetic Predisposition To Colorectal Cancer. Nature Reviews Cancer, 4**,** 769-780.

Deleu, M. & Paquot, M. 2004. From Renewable Vegetables Resources To Microorganisms: New Trends In Surfactants. Comptes Rendus Chimie, 7**,** 641-646.

De Oliveira, M. R., Magri, A., Baldo, C., Camilios-Neto, D., Minucelli, T. & Celligoi, M. A. P. C. 2015. Review: Sophorolipids A Promising Biosurfactant And It's Applications.
Demain, A. L. & Vaishnav, P. 2011. Natural Products For Cancer Chemotherapy. Microbial Biotechnology, 4**,** 687-699.

De Rienzo, M. A. D. & Martin, P. J. 2016. Effect Of Mono And Di-Rhamnolipids On Biofilms Pre-Formed By Bacillus Subtilis. Current Microbiology, 73**,** 183-189.

De Rienzo, M. A. D., Banat, I. M., Dolman, B., Winterburn, J. & Martin, P. J. 2015. Sophorolipid Biosurfactants: Possible Uses As Antibacterial And Antibiofilm Agent. New Biotechnology, 32**,** 720-726.

De Robertis, M., Massi, E., Poeta, M. L., Carotti, S., Morini, S., Cecchetelli, L., Signori, E. & Fazio, V. M. 2011. The Aom/Dss Murine Model For The Study Of Colon Carcinogenesis: From Pathways To Diagnosis And Therapy Studies. Journal Of Carcinogenesis, 10**,** 9.

Develter, D. W. & Lauryssen, L. M. 2010. Properties And Industrial Applications Of Sophorolipids. European Journal Of Lipid Science And Technology, 112**,** 628-638.

D'incalci, M., Steward, W. P. & Gescher, A. J. 2005. Use Of Cancer Chemopreventive Phytochemicals As Antineoplastic Agents. The Lancet Oncology, 6**,** 899-904.

Dhar, S., Reddy, E. M., Prabhune, A., Pokharkar, V., Shiras, A. & Prasad, B. 2011. Cytotoxicity Of Sophorolipid-Gellan Gum-Gold Nanoparticle Conjugates And Their Doxorubicin Loaded Derivatives Towards Human Glioma And Human Glioma Stem Cell Lines. Nanoscale, 3**,** 575-580.

Dianzani, C., Minelli, R., Gigliotti, C. L., Occhipinti, S., Giovarelli, M., Conti, L., Boggio, E., Shivakumar, Y., Baldanzi, G. & Malacarne, V. 2014. B7h Triggering Inhibits The Migration Of Tumor Cell Lines. The Journal Of Immunology, 192**,** 4921-4931.

Ducreux, J., Ballerini, D., Baviere, M., Bocard, C. & Monin, N. 1997. Composition Containing A Surface Active Compound And Glycolipids And Decontamination Process For A Porous Medium Polluted By Hydrocarbons. Google Patents.

Dubois, R. N., Radhika, A., Reddy, B. S. & Entingh, A. J. 1996. Increased Cyclooxygenase-2 Levels In Carcinogen-Induced Rat Colonic Tumors. Gastroenterology, 110**,** 1259-1262.

Dunlop, M. G., Farrington, S. M., Carothers, A. D., Wyllie, A. H., Sharp, L., Burn, J.,

Liu, B., Kinzler, K. W. & Vogelstein, B. 1997. Cancer Risk Associated With Germline Dna Mismatch Repair Gene Mutations. Human Molecular Genetics, 6**,** 105-110

Eckel, F. & Schmid, R. M. 2014. Chemotherapy And Targeted Therapy In Advanced Biliary Tract Carcinoma: A Pooled Analysis Of Clinical Trials. Chemotherapy, 60**,** 13-23.

Ehehalt, R., Keller, P., Haass, C., Thiele, C. & Simons, K. 2003. Amyloidogenic Processing Of The Alzheimer B-Amyloid Precursor Protein Depends On Lipid Rafts. The Journal Of Cell Biology, 160**,** 113-123.

Faivre, V. & Rosilio, V. 2010. Interest Of Glycolipids In Drug Delivery: From Physicochemical Properties To Drug Targeting. Expert Opinion On Drug Delivery, 7**,** 1031-1048.

Fearon, E. R. & Vogelstein, B. 1990. A Genetic Model For Colorectal Tumorigenesis. Cell, 61**,** 759-767.

Fidler, I. J. 2003. The Pathogenesis Of Cancer Metastasis: The'seed And Soil'hypothesis Revisited. Nature Reviews Cancer, 3**,** 453-458.

Fiers, W., Beyaert, R., Declercq, W. & Vandenabeele, P. 1999. More Than One Way To Die: Apoptosis, Necrosis And Reactive Oxygen Damage. Oncogene, 18**,** 7719-7730.

Finnerty, W. R. 1994. Biosurfactants In Environmental Biotechnology. Current Opinion In Biotechnology, 5**,** 291-295.

Fishel, R., Lescoe, M. K., Rao, M., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M. & Kolodner, R. 1993. The Human Mutator Gene Homolog Msh2 And Its Association With Hereditary Nonpolyposis Colon Cancer. Cell, 75**,** 1027-1038.

Flam, F. 1994. Race To Synthesize Taxol Ends In A Tie. Science, 263**,** 911-912.

Fleming, M., Ravula, S., Tatishchev, S. F. & Wang, H. L. 2012. Colorectal Carcinoma: Pathologic Aspects. Journal Of Gastrointestinal Oncology, 3**,** 153-173.

Fodde, R. 2002. The Apc Gene In Colorectal Cancer. European Journal Of Cancer, 38**,** 867-871.

Fodde, R., Edelmann, W., Yang, K., Van Leeuwen, C., Carlson, C., Renault, B., Breukel, C., Alt, E., Lipkin, M. & Khan, P. M. 1994. A Targeted Chain-Termination Mutation In The Mouse Apc Gene Results In Multiple Intestinal Tumors. Proceedings Of The National Academy Of Sciences, 91**,** 8969-8973.

Fogh, J. & Trempe, G. 1975. New Human Tumor Cell Lines. Human Tumor Cells In Vitro. Springer.

Foulds, L. 1965. Multiple Etiologic Factors In Neoplastic Development. Cancer Research, 25**,** 1339-1347.

Fracchia, L., Cavallo, M., Martinotti, M. G. & Banat, I. M. 2012. Biosurfactants And Bioemulsifiers Biomedical And Related Applications–Present Status And Future

Potentials. Biomedical Science, Engineering And Technology, 14**,** 326-335.

Fracchia, L., Banat, I. M., Martinotti, M. G. & Cavallo, M. 2012. Biosurfactants And Bioemulsifiers Biomedical And Related Applications-Present Status And Future Potentials, Intech Open Access Publisher.

Fracchia, L., Banat, J. J., Cavallo, M., Ceresa, C. & Banat, I. 2015. Potential Therapeutic Applications Of Microbial Surface-Activecompounds. Aims Bioengineering, 2**,** 144-162.

Fu, S. L., Wallner, S. R., Bowne, W. B., Hagler, M. D., Zenilman, M. E., Gross, R. & Bluth, M. H. 2008. Sophorolipids And Their Derivatives Are Lethal Against Human Pancreatic Cancer Cells. Journal Of Surgical Research, 148**,** 77-82.

Gajate, C., Gonzalez-Camacho, F. & Mollinedo, F. 2009. Involvement Of Raft Aggregates Enriched In Fas/Cd95 Death-Inducing Signaling Complex In The Antileukemic Action Of Edelfosine In Jurkat Cells. Plos One, 4**,** E5044.

Gao, Q., Wang, X. Y., Zhou, J. & Fan, J. 2011. Cell Line Misidentification: The Case Of The Chang Liver Cell Line. Hepatology, 54**,** 1894-1895.

Garti, N. 2002. Food Emulsifiers: Structure–Reactivity Relationships, Design, And Applications. Physical Properties Of Lipids**,** 265.

Gajate, C. & Mollinedo, F. 2007. Edelfosine And Perifosine Induce Selective Apoptosis In Multiple Myeloma By Recruitment Of Death Receptors And Downstream Signaling Molecules Into Lipid Rafts. Blood, 109**,** 711-719.

Gajate, C. & Mollinedo, F. 2014. Lipid Rafts, Endoplasmic Reticulum And Mitochondria In The Antitumor Action Of The Alkylphospholipid Analog Edelfosine. Anti-Cancer Agents In Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 14**,** 509-527.

Gerhard, B. & Heinz, S. 1940. Process Of Preparing Cyclic Carboxylic Acids Substituted In The Nucleus. Google Patents.

Giardiello, F. M., Allen, J. I., Axilbund, J. E., Boland, C. R., Burke, C. A., Burt, R. W., Church, J. M., Dominitz, J. A., Johnson, D. A. & Kaltenbach, T. 2014. Guidelines On Genetic Evaluation And Management Of Lynch Syndrome: A Consensus Statement By The Us Multi-Society Task Force On Colorectal Cancer. Gastroenterology, 147**,** 502-526.

Giessler-Blank, S., Schilling, M., Thum, O. & Sieverding, E. 2010. Use Of Sophorolipids And Derivatives Thereof In Combination With Pesticides As Adjuvant/Additive For Plant Protection And The Industrial Non-Crop Field. Google Patents.

Giovannucci, E., Rimm, E. B., Ascherio, A., Stampfer, M. J., Colditz, G. A. & Willett, W. C. 1995. Alcohol, Low-Methionine-Low-Folate Diets, And Risk Of Colon Cancer In Men. Journal Of The National Cancer Institute, 87**,** 265-273.

Goodlad, R., Ryan, A., Wedge, S., Pyrah, I., Alferez, D., Poulsom, R., Smith, N., Mandir, N., Watkins, A. & Wilkinson, R. 2006. Inhibiting Vascular

Endothelial Growth Factor Receptor-2 Signaling Reduces Tumor Burden In The Apcmin/+ Mouse Model Of Early Intestinal Cancer. Carcinogenesis, 27**,** 2133-2139.

Gottardi, C. J., Wong, E. & Gumbiner, B. M. 2001. E-Cadherin Suppresses Cellular Transformation By Inhibiting B-Catenin Signaling In An Adhesion-Independent Manner. The Journal Of Cell Biology, 153**,** 1049-1060.

Gniadecki, R., Christoffersen, N. & Wulf, H. C. 2002. Cholesterol-Rich Plasma Membrane Domains (Lipid Rafts) In Keratinocytes: Importance In The Baseline And Uva-Induced Generation Of Reactive Oxygen Species. Journal Of Investigative Dermatology, 118**,** 582-588.

Gorin, P., Spencer, J. & Tulloch, A. 1961. Hydroxy Fatty Acid Glycosides Of Sophorose From Torulopsis Magnoliae. Canadian Journal Of Chemistry, 39**,** 846-855.

Gould, T. D., Gray, N. A. & Manji, H. K. 2003. Effects Of A Glycogen Synthase Kinase-Inhibitor, Lithium, In Adenomatous Polyposis Coli Mutant Mice. Pharmacological Research, 48**,** 49-53.

Graziadei, L., Riabowol, K. & Bar-Sagi, D. 1990. Co-Capping Of Ras Proteins With Surface Immunoglobulins In B Lymphocytes. Nature, 347**,** 396.

Groot, R. D. & Rabone, K. 2001. Mesoscopic Simulation Of Cell Membrane Damage, Morphology Change And Rupture By Nonionic Surfactants. Biophysical Journal, 81**,** 725-736.

Gross, R., Shah, V. & Doncel, G. 2004. Spermicidal And Virucidal Properties Of Various Forms Of Sophorolipids. Google Patents.

George, K. S. & Wu, S. 2012. Lipid Raft: A Floating Island Of Death Or Survival. Toxicology And Applied Pharmacology, 259**,** 311-319.

Hagler, M., Smith-Norowitz, T., Chice, S., Wallner, S., Viterbo, D., Mueller, C., Gross, R., Nowakowski, M., Schulze, R. & Zenilman, M. 2007. Sophorolipids Decrease Ige Production In U266 Cells By Downregulation Of Bsap (Pax5), Tlr-2, Stat3 And Il-6. Journal Of Allergy And Clinical Immunology, 119**,** S263.

Hanahan, D. & Weinberg, R. A. 2011. Hallmarks Of Cancer: The Next Generation. Cell, 144**,** 646-674.

Harder, T., Scheiffele, P., Verkade, P. & Simons, K. 1998. Lipid Domain Structure Of The Plasma Membrane Revealed By Patching Of Membrane Components. The Journal Of Cell Biology, 141**,** 929-942.

Hardin, R., Pierre, J., Schulze, R., Mueller, C. M., Fu, S. L., Wallner, S. R., Stanek, A., Shah, V., Gross, R. A. & Weedon, J. 2007. Sophorolipids Improve Sepsis Survival: Effects Of Dosing And Derivatives. Journal Of Surgical Research, 142**,** 314-319.

Harley, B. A., Kim, H.-D., Zaman, M. H., Yannas, I. V., Lauffenburger, D. A. & Gibson, L. J. 2008. Microarchitecture Of Three-Dimensional Scaffolds Influences Cell Migration Behavior Via Junction Interactions. Biophysical Journal, 95**,** 4013-4024.

Harrison, S. J., Mainwaring, P., Price, T., Millward, M. J., Padrik, P., Underhill, C. R., Cannell, P. K., Reich, S. D., Trikha, M. & Spencer, A. 2016. Phase I Clinical Trial Of Marizomib (Npi-0052) In Patients With Advanced Malignancies Including Multiple Myeloma: Study Npi-0052-102 Final Results. Clinical Cancer Research, 22**,** 4559-4566.

Harvey, A. L. 2008. Natural Products In Drug Discovery. Drug Discovery Today, 13**,** 894-901.

Hasty, P., Livi, C. B., Dodds, S. G., Jones, D., Strong, R., Javors, M., Fischer, K. E., Sloane, L., Murthy, K. & Hubbard, G. 2014. Erapa Restores A Normal Life Span In A Fap Mouse Model. Cancer Prevention Research, 7**,** 169-178.

Hawk, N. N., Long, T.-E., Imam, M. H., Mathew, B. M., Kim, S., Chen, Z., Goodman,M., Sullivan, P., Brutcher, E. & Kauh, J. 2015. Clinicopathologic Features And Outcome Of Young Adults With Stage Iv Colorectal Cancer. American Journal Of Clinical Oncology, 38**,** 543-549.

Head, B. P., Patel, H. H. & Insel, P. A. 2014. Interaction Of Membrane/Lipid Rafts With The Cytoskeleton: Impact On Signaling And Function: Membrane/Lipid Rafts, Mediators Of Cytoskeletal Arrangement And Cell Signaling. Biochimica Et Biophysica Acta (Bba)-Biomembranes, 1838**,** 532-545.

Heerklotz, H. 2001. Membrane Stress And Permeabilization Induced By Asymmetric Incorporation Of Compounds. Biophysical Journal, 81**,** 184-195.

Heerklotz, H. 2002. Triton Promotes Domain Formation In Lipid Raft Mixtures. Biophysical Journal, 83**,** 2693-2701.

Heerklotz, H. 2008. Interactions Of Surfactants With Lipid Membranes. Quarterly Reviews Of Biophysics, 41**,** 205-264.

Heerklotz, H., Binder, H., Lantzsch, G., Klose, G. & Blume, A. 1997. Lipid/Detergent Interaction Thermodynamics As A Function Of Molecular Shape. The Journal Of Physical Chemistry B, 101**,** 639-645.

Heerklotz, H. & Seelig, J. 2007. Leakage And Lysis Of Lipid Membranes Induced By The Lipopeptide Surfactin. European Biophysics Journal, 36**,** 305-314.

Hendrich, A. & Michalak, K. 2003. Lipids As A Target For Drugs Modulating Multidrug Resistance Of Cancer Cells. Current Drug Targets, 4**,** 23-30.

Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J.-P. J., Markowitz, S., Willson, J. K., Hamilton, S. R. & Kinzler, K. W. 1998. Incidence And Functional Consequences Of Hmlh1 Promoter Hypermethylation In Colorectal Carcinoma. Proceedings Of The National Academy Of Sciences, 95**,** 6870-6875.

Higuchi, M., Honda, T., Proske, R. J. & Yeh, E. T. 1998. Regulation Of Reactive Oxygen Species-Induced Apoptosis And Necrosis By Caspase 3-Like Proteases. Oncogene, 17**,** 2753-2760.

Hoffman, R. M. 2015. Patient-Derived Orthotopic Xenografts: Better Mimic Of Metastasis Than Subcutaneous Xenografts. Nature Reviews. Cancer, 15**,** 451.

Holcomb, V. B., Rodier, F., Choi, Y., Busuttil, R. A., Vogel, H., Vijg, J., Campisi, J. & Hasty, P. 2008. Ku80 Deletion Suppresses Spontaneous Tumors And Induces A P53-Mediated Dna Damage Response. Cancer Research, 68**,** 9497-9502.

Holliday, R. & Tarrant, G. 1972. Altered Enzymes In Ageing Human Fibroblasts. Nature, 238**,** 26.

Hu, Y. & Ju, L.-K. 2001. Purification Of Lactonic Sophorolipids By Crystallization. Journal Of Biotechnology, 87**,** 263-272.

Huang, Q., Shen, H.-M., Shui, G., Wenk, M. R. & Ong, C.-N. 2006. Emodin Inhibits Tumor Cell Adhesion Through Disruption Of The Membrane Lipid Raft-Associated Integrin Signaling Pathway. Cancer Research, 66**,** 5807-5815.

Ignatenko, N. A., Holubec, H., Besselsen, D. G., Blohm-Mangone, K. A., Padilla-Torres, J., Nagle, R. B., De Alboránç, I. M., Guillen-R, J. M. & Gerner, E. W. 2006. Role Of C-Myc In Intestinal Tumorigenesis Of The Apc Min/+ Mouse1. Cancer Biology & Therapy, 5**,** 1658-1664.

Ikeda, Y., Sunakawa, T., Tsuchiya, S., Kondo, M. & Okamoto, K. 1986. [Toxicological Studies On Sophorolipid Derivatives.(I). Acute Toxicity, Eye Irritation, Primary Skin Irritation, Skin Sensitization, Phototoxicity, Photosensitization, Mutagenicity Of Polyoxypropylene (12)[(2'-0-Beta-D-Glucopyranosyl-Beta-D-Glucopyranosyl) Oxy-] Fatty Acid Ester-]. The Journal Of Toxicological Sciences, 11**,** 197-211.

Ishii, N., Kobayashi, T., Matsumiya, K., Ryu, M., Hirata, Y., Matsumura, Y. & Suzuki, Y. A. 2012. Transdermal Administration Of Lactoferrin With Sophorolipid 1 1 This Article Is Part Of A Special Issue Entitled Lactoferrin And Has Undergone The Journal's Usual Peer Review Process. Biochemistry And Cell Biology, 90**,** 504-512.

Imura, T., Morita, T., Fukuoka, T., Ryu, M., Igarashi, K., Hirata, Y. & Kitamoto, D. 2013. Spontaneous Vesicle Formation From Sodium Salt Of Acidic Sophorolipid And Its Application As A Skin Penetration Enhancer. Journal Of Oleo Science, 63**,** 141-147.

Itoh, M., Nagafuchi, A., Moroi, S. & Tsukita, S. 1997. Involvement Of Zo-1 In Cadherin-Based Cell Adhesion Through Its Direct Binding To A Catenin And Actin Filaments. The Journal Of Cell Biology, 138**,** 181-192.

Janek, T., Krasowska, A., Radwańska, A. & Łukaszewicz, M. 2013. Lipopeptide Biosurfactant Pseudofactin Ii Induced Apoptosis Of Melanoma A 375 Cells By Specific Interaction With The Plasma Membrane. Plos One, 8**,** E57991.

Ji, C. & Kaplowitz, N. 2003. Betaine Decreases Hyperhomocysteinemia, Endoplasmic Reticulum Stress, And Liver Injury In Alcohol-Fed Mice. Gastroenterology, 124**,** 1488-1499.

Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovic, K., Tutsch, K., Cole, C. E., Lubet, R. A., Kelloff, G. J., Verma, A. & Moser, A. R. 1996. Chemoprevention Of Spontaneous Intestinal Adenomas In The Apc Min Mouse Model By The Nonsteroidal Anti-Inflammatory Drug Piroxicam. Cancer Research, 56**,** 710-714.

Jacoby, R. F., Seibert, K., Cole, C. E., Kelloff, G. & Lubet, R. A. 2000. The Cyclooxygenase-2 Inhibitor Celecoxib Is A Potent Preventive And Therapeutic Agent In The Min Mouse Model Of Adenomatous Polyposis. Cancer Research, 60**,** 5040-5044.

Jacobson, M. R. & Bemiller, J. N. 1998. Method For Determining The Rate And Extent Of Accelerated Starch Retrogradation 1. Cereal Chemistry, 75**,** 22-29.

Johnson, R. L. & Fleet, J. C. 2013. Animal Models Of Colorectal Cancer. Cancer And Metastasis Reviews, 32**,** 39-61.

Jonas, S., Windeatt, S., O-Boateng, A., Fordy, C. & Allen-Mersh, T. 1996. Identification Of Carcinoembryonic Antigen-Producing Cells Circulating In The Blood Of Patients With Colorectal Carcinoma By Reverse Transcriptase Polymerase Chain Reaction. Gut, 39**,** 717-721.

Joshi-Navare, K., Shiras, A. & Prabhune, A. 2011. Differentiation-Inducing Ability Of Sophorolipids Of Oleic And Linoleic Acids Using A Glioma Cell Line. Biotechnology Journal, 6**,** 509-512.

Joshi-Navare, K. & Prabhune, A. 2013. A Biosurfactant-Sophorolipid Acts In Synergy With Antibiotics To Enhance Their Efficiency. Biomed Research International, 2013.

Jucá, M. J., Bandeira, B. C., Carvalho, D. S. & Leal, A. T. 2014. Comparative Study Of 1, 2-Dimethylhydrazine And Azoxymethane On The Induction Of Colorectal Cancer In Rats. Journal Of Coloproctology, 34**,** 167-173.

Justin, R. Y., Huang, Q., Hou, P. & Lai, J.-P. 2015. Collision Tumor Of Colonic Adenocarcinoma And Ebv-Driven Large B-Cell Lymphoma: A Case Report And Review Of Literature. Cancer Treatment Communications, 3**,** 7-12.

K Morya, V., Ahn, C., Jeon, S. & Kim, E.-K. 2013. Medicinal And Cosmetic Potentials Of Sophorolipids. Mini Reviews In Medicinal Chemistry, 13**,** 1761-1768.

Kang, S.-Y., Seeram, N. P., Nair, M. G. & Bourquin, L. D. 2003. Tart Cherry Anthocyanins Inhibit Tumor Development In Apc Min Mice And Reduce Proliferation Of Human Colon Cancer Cells. Cancer Letters, 194**,** 13-19.

Kang, S.-W., Kim, Y.-B., Shin, J.-D. & Kim, E.-K. 2010. Enhanced Biodegradation Of Hydrocarbons In Soil By Microbial Biosurfactant, Sophorolipid. Applied Biochemistry And Biotechnology, 160**,** 780-790.

Karim, B. O. & Huso, D. L. 2013. Mouse Models For Colorectal Cancer. Am J Cancer Res, 3**,** 240-250.

Kelloff, G. J., Boone, C. W., Crowell, J. A., Steele, V. E., Lubet, R. & Sigman, C. C. 1994. Chemopreventive Drug Development: Perspectives And Progress. Cancer

Epidemiology And Prevention Biomarkers, 3**,** 85-98.

Kiesslich, R., Burg, J., Vieth, M., Gnaendiger, J., Enders, M., Delaney, P., Polglase, A., Mclaren, W., Janell, D. & Thomas, S. 2004. Confocal Laser Endoscopy For Diagnosing Intraepithelial Neoplasias And Colorectal Cancer In Vivo. Gastroenterology, 127**,** 706-713.

Kimmey, M. B. 2004. Endoscopic Methods (Other Than Stents) For Palliation Of Rectal Carcinoma. Journal Of Gastrointestinal Surgery, 8**,** 270-273.

Kim, S.-Y., Kim, J. Y., Kim, S.-H., Bae, H. J., Yi, H., Yoon, S. H., Koo, B. S., Kwon, M., Cho, J. Y. & Lee, C.-E. 2007. Surfactin From Bacillus Subtilis Displays Anti-Proliferative Effect Via Apoptosis Induction, Cell Cycle Arrest And Survival Signaling Suppression. Febs Letters, 581**,** 865-871.

Koley, D. & Bard, A. J. 2010. Triton X-100 Concentration Effects On Membrane Permeability Of A Single Hela Cell By Scanning Electrochemical Microscopy (Secm). Proceedings Of The National Academy Of Sciences, 107**,** 16783-16787.

Knudson, A. G. 1996. Hereditary Cancer: Two Hits Revisited. Journal Of Cancer Research And Clinical Oncology, 122**,** 135-140.

Kondo, Y. & Issa, J.-P. J. 2004. Epigenetic Changes In Colorectal Cancer. Cancer And Metastasis Reviews, 23**,** 29-39.

Krishnaiah, Y. S. 2010. Pharmaceutical Technologies For Enhancing Oral Bioavailability Of Poorly Soluble Drugs. J Bioequiv Availab, 2**,** 28-36.

Kumar, V., Abbas, A. K. & Aster, J. C. 2012. Robbins Basic Pathology, Elsevier Health Sciences.

Kralova, I. & Sjöblom, J. 2009. Surfactants Used In Food Industry: A Review. Journal Of Dispersion Science And Technology, 30**,** 1363-1383.

Krishnamachary, B., Berg-Dixon, S., Kelly, B., Agani, F., Feldser, D., Ferreira, G., Iyer, N., Larusch, J., Pak, B. & Taghavi, P. 2003. Regulation Of Colon Carcinoma Cell Invasion By Hypoxia-Inducible Factor 1. Cancer Research, 63**,** 1138-1143.

Kume, G., Gallotti, M. & Nunes, G. 2008. Review On Anionic/Cationic Surfactant Mixtures. Journal Of Surfactants And Detergents, 11**,** 1-11.

Lacour, S., Hammann, A., Grazide, S., Lagadic-Gossmann, D., Athias, A., Sergent, O., Laurent, G., Gambert, P., Solary, E. & Dimanche-Boitrel, M.-T. 2004. Cisplatin-Induced Cd95 Redistribution Into Membrane Lipid Rafts Of Ht29 Human Colon Cancer Cells. Cancer Research, 64**,** 3593-3598.

Lang, S., Brakemeier, A., Heckmann, R., Spöckner, S. & Rau, U. 2000. Production Of Native And Modified Sophorose Lipids. Chimica Oggi, 18**,** 76-79.

Lang, S. & Wullbrandt, D. 1999. Rhamnose Lipids–Biosynthesis, Microbial Production And Application Potential. Applied Microbiology And Biotechnology, 51**,** 22-32.

Laing, J. G., Chou, B. C. & Steinberg, T. H. 2005. Zo-1 Alters The Plasma Membrane Localization And Function Of Cx43 In Osteoblastic Cells. Journal Of Cell Science, 118**,** 2167-2176.

Lambert, D., O'neill, C. A. & Padfield, P. J. 2008. Methyl-B-Cyclodextrin Increases Permeability Of Caco-2 Cell Monolayers By Displacing Specific Claudins From Cholesterol Rich Domains Associated With Tight Junctions. Cellular Physiology And Biochemistry, 20**,** 495-506.

Lefebvre, A.-M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J.-C., Geboes, K., Briggs, M., Heyman, R. & Auwerx, J. 1998. Activation Of The Peroxisome Proliferator-Activated Receptor Γ Promotes The Development Of Colon Tumors In C57bl/6j-Apcmin/+ Mice. Nature Medicine, 4**,** 1053-1057.

Lengauer, C., Kinzler, K. W. & Vogelstein, B. 1997. Dna Methylation And Genetic Instability In Colorectal Cancer Cells. Proceedings Of The National Academy Of Sciences, 94**,** 2545-2550.

Levin, J. & Miller, R. 2011. A Guide To The Ingredients And Potential Benefits Of Over-The-Counter Cleansers And Moisturizers For Rosacea Patients. J Clin Aesthet Dermatol, 4**,** 31-49.

Li, H., Guo, W., Ma, X.-J., Li, J.-S. & Song, X. 2016. In Vitro And In Vivo Anticancer Activity Of Sophorolipids To Human Cervical Cancer. Applied Biochemistry And Biotechnology**,** 1-16.

Li, J., Li, H., Li, W., Xia, C. & Song, X. 2016b. Identification And Characterization Of A Flavin-Containing Monooxygenase Moa And Its Function In A Specific Sophorolipid Molecule Metabolism In Starmerella Bombicola. Applied Microbiology And Biotechnology, 100**,** 1307-1318.

Li, H., Guo, W., Ma, X.-J., Li, J.-S. & Song, X. 2016. In Vitro And In Vivo Anticancer Activity Of Sophorolipids To Human Cervical Cancer. Applied Biochemistry And Biotechnology**,** 1-16.

Li, Y.-H. & Zhu, C. 1999. A Modified Boyden Chamber Assay For Tumor Cell Transendothelial Migration In Vitro. Clinical & Experimental Metastasis, 17**,** 423-429.

Li, Y. , Park, M. J., Ye, S.-K., Kim, C.-W. & Kim, Y.-N. 2006. Elevated Levels Of Cholesterol-Rich Lipid Rafts In Cancer Cells Are Correlated With Apoptosis Sensitivity Induced By Cholesterol-Depleting Agents. The American Journal Of Pathology, 168**,** 1107-1118.

Lievre, A., Bachet, J.-B., Le Corre, D., Boige, V., Landi, B., Emile, J.-F., Côté, J.-F., Tomasic, G., Penna, C. & Ducreux, M. 2006. Kras Mutation Status Is Predictive Of Response To Cetuximab Therapy In Colorectal Cancer. Cancer Research, 66**,** 3992-3995.

Liu, B., Ezeogu, L., Zellmer, L., Yu, B., Xu, N. & Joshua Liao, D. 2015. Protecting The Normal In Order To Better Kill The Cancer. Cancer Medicine, 4**,** 1394-1403.

Loeb, L. A. 1991. Mutator Phenotype May Be Required For Multistage Carcinogenesis. Cancer Res, 51.

Longley, D. B., Harkin, D. P. & Johnston, P. G. 2003. 5-Fluorouracil: Mechanisms Of Action And Clinical Strategies. Nature Reviews Cancer, 3**,** 330-338.

Liu, K., Liu, P.-C., Liu, R. & Wu, X. 2015. Dual Ao/Eb Staining To Detect Apoptosis In Osteosarcoma Cells Compared With Flow Cytometry. Medical Science Monitor Basic Research, 21**,** 15.

LIU, Y. & Bodmer, W. F. 2006. Analysis of P53 mutations and their expression in 56 colorectal cancer cell lines. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 976-981.

Lupton, S. J., Huwe, J. K., Smith, D. J., Dearfield, K. L. & Johnston, J. J. 2014. Distribution And Excretion Of Perfluorooctane Sulfonate (Pfos) In Beef Cattle (Bos Taurus). Journal Of Agricultural And Food Chemistry, 62**,** 1167-1173.

Lydon, H. L., Baccile, N., Callaghan, B., Marchant, R., Mitchell, C. A. & Banat, I. M. 2017. Adjuvant Antibiotic Activity Of Acidic Sophorolipids With Potential For Facilitating Wound Healing. Antimicrobial Agents And Chemotherapy**,** Aac. 02547-16.

Lynch, H. T. & Lynch, J. F. 1985. Hereditary Nonpolyposis Colorectal Cancer (Lynch Syndromes I And Ii): A Common Genotype Linked To Oncogenes? Medical Hypotheses, 18**,** 19-28.

Ma, X., Li, H. & Song, X. 2012. Surface And Biological Activity Of Sophorolipid Molecules Produced By Wickerhamiella Domercqiae Var. Sophorolipid Cgmcc 1576. Journal Of Colloid And Interface Science, 376**,** 165-172.

Madison, B. B., Dunbar, L., Qiao, X. T., Braunstein, K., Braunstein, E. & Gumucio, D. L. 2002. Cis Elements Of The Villin Gene Control Expression In Restricted Domains Of The Vertical (Crypt) And Horizontal (Duodenum, Cecum) Axes Of The Intestine. Journal Of Biological Chemistry, 277**,** 33275-33283.

Maekawa, A., Onodera, H., Kanno, J., Furuta, K., Nagaoka, T., Todate, A., Matsushima, Y., Oh-Hara, T. & Kawazoe, Y. 1988. Carcinogenicity And Organ Specificity Of N-Trimethylsilylmethyl-N-Nitrosoure (Tms-Mnu), N-Neopentyl-N-Nitrosourea (Neopnu), And N-Methyl-N-Nitrosourea (Mnu) In Rats. Journal Of Cancer Research And Clinical Oncology, 114**,** 473-476.

Maingault, M. 1999. Utilization Of Sophorolipids As Therapeutically Active Substances Or Cosmetic Products, In Particular For The Treatment Of The Skin. Google Patents.

Manzke, C. 1999. Prozeßabha¨Ngige Aufarbeitung Und Hplcgestu¨Tze Analyse Von Sophoroselipiden Aus Candida Bombicola.

Marchant, R. & Banat, I. M. 2012. Biosurfactants: A Sustainable Replacement For Chemical Surfactants? Biotechnology Letters, 34**,** 1597-1605.

Marusiak, A. A., Stephenson, N. L., Baik, H., Trotter, E. W., Li, Y., Blyth, K., Mason, S., Chapman, P., Puto, L. A. & Read, J. A. 2016. Recurrent Mlk4 Loss-Of-Function Mutations Suppress Jnk Signaling To Promote Colon Tumorigenesis. Cancer Research, 76**,** 724-735.

Masters, J. R. 2002. Hela Cells 50 Years On: The Good, The Bad And The Ugly. Nature Reviews Cancer, 2**,** 315-319.

Mazevet, M., Moulin, M., Llach-Martinez, A., Chargari, C., Deutsch, É., Gomez, A.-M. & Morel, É. 2013. Complications Of Chemotherapy, A Basic Science Update. La Presse Médicale, 42**,** E352-E361.

Mccart, A. E., Vickaryous, N. K. & Silver, A. 2008. Apc Mice: Models, Modifiers And Mutants. Pathology-Research And Practice, 204**,** 479-490.

Mcconnell, R. 1976. Familial Polyposis Coli. Family Studies, Histopathology, Differential Diagnosis, And Results Of Treatment. Journal Of Medical Genetics, 13**,** 415.

Mcquade, R. M., Bornstein, J. C. & Nurgali, K. 2014. Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea: Current Treatments And Side-Effects. International Journal Of Clinical Medicine, 2014.

Mcintyre, R. E., Buczacki, S. J., Arends, M. J. & Adams, D. J. 2015. Mouse Models Of Colorectal Cancer As Preclinical Models. Bioessays, 37**,** 909-920.

Meyskens, F. L., Mclaren, C. E., Pelot, D., Fujikawa-Brooks, S., Carpenter, P. M., Hawk, E., Kelloff, G., Lawson, M. J., Kidao, J. & Mccracken, J. 2008. Difluoromethylornithine Plus Sulindac For The Prevention Of Sporadic Colorectal Adenomas: A Randomized Placebo-Controlled, Double-Blind Trial. Cancer Prevention Research, 1**,** 32-38.

Mohelnikova-Duchonova, B., Melichar, B. & Soucek, P. 2014. Folfox/Folfiri Pharmacogenetics: The Call For A Personalized Approach In Colorectal Cancer Therapy. World Journal Of Gastroenterology: Wjg, 20**,** 10316.

Mollinedo, F. & Gajate, C. 2010. Lipid Rafts And Clusters Of Apoptotic Signaling Molecule-Enriched Rafts In Cancer Therapy. Future Oncology, 6**,** 811-821.

Montoneri, E., Boffa, V., Savarino, P., Tambone, F., Adani, F., Micheletti, L., Gianotti, C. & Chiono, R. 2009. Use Of Biosurfactants From Urban Wastes Compost In Textile Dyeing And Soil Remediation. Waste Management, 29**,** 383-389.

Morgan, M. A. & Rubin, S. C. 1998. Long-Term Complications Of Chemotherapy. Primary Care Update For Ob/Gyns, 5**,** 65-68.

Moser, A., Luongo, C., Gould, K. A., Mcneley, M., Shoemaker, A. & Dove, W. 1995. Apcmin: A Mouse Model For Intestinal And Mammary Tumorigenesis. European Journal Of Cancer, 31**,** 1061-1064.

Moser, A. R., Mattes, E. M., Dove, W. F., Lindstrom, M. J., Haag, J. D. & Gould, M. N. 1993. Apcmin, A Mutation In The Murine Apc Gene, Predisposes To Mammary

Carcinomas And Focal Alveolar Hyperplasias. Proceedings Of The National Academy Of
Sciences, 90**,** 8977-8981.

Mueller, C. M., Lin, Y.-Y., Viterbo, D., Pierre, J., Murray, S. A., Shah, V., Gross, R., Schulze, R., Zenilman, M. E. & Bluth, M. H. 2006. Sophorolipid Treatment Decreases Inflammatory Cytokine Expression In An In Vitro Model Of Experimental Sepsis. The Faseb Journal, 20**,** A204.

Mulligan, C. N. 2005. Environmental Applications For Biosurfactants. Environmental Pollution, 133**,** 183-198.

Mukherjee, S., Das, P. & Sen, R. 2006. Towards Commercial Production Of Microbial Surfactants. Trends In Biotechnology, 24**,** 509-515.

Murai, T. 2011. The Role Of Lipid Rafts In Cancer Cell Adhesion And Migration. International Journal Of Cell Biology, 2012.

Nakagawa, H., Nuovo, G. J., Zervos, E. E., Martin, E. W., Salovaara, R., Aaltonen, L. A. & De La Chapelle, A. 2001. Age-Related Hypermethylation Of The 5′ Region Of Mlh1 In Normal Colonic Mucosa Is Associated With Microsatellite-Unstable Colorectar

Cancer Development. Cancer Research, 61**,** 6991-6995.

Napolitano, L. M. 2006. Sophorolipids In Sepsis: Antiinflammatory Or Antibacterial? Critical Care Medicine, 34**,** 258-259.

Narisawa, T., Magadia, N. E., Weisburger, J. H. & Wynder, E. L. 1974. Promoting Effect Of Bile Acids On Colon Carcinogenesis After Intrarectal Instillation Of N-Methyl-N′ Nitro-N-Nitrosoguanidine In Rats. Journal Of The National Cancer Institute, 53**,** 1093-1097.

Näthke, I. 2004. Apc At A Glance. Journal Of Cell Science, 117**,** 4873-4875.

Nerurkar, A. S., Suthar, H. G. & Desai, A. J. 2012. Biosystem Development For Microbial Enhanced Oil Recovery (Meor). Microorganisms In Sustainable Agriculture And Biotechnology. Springer.

Newcomb, E. W., Steinberg, J. J. & Pellicer, A. 1988. Ras Oncogenes And Phenotypic Staging In N-Methylnitrosourea-And Γ-Irradiation-Induced Thymic Lymphomas In C57bl/6j Mice. Cancer Research, 48**,** 5514-5521.

Newman, D. & Shapiro, S. 2008. Microbial Prescreens For Anticancer Activity. Sim News, 58**,** 132-150.

Neugut, A. I., Jacobson, J. S., Suh, S., Mukherjee, R. & Arber, N. 1998. The Epidemiology Of Cancer Of The Small Bowel. Cancer Epidemiology Biomarkers & Prevention, 7**,** 243-251.

Newmark, H. L., Lipkin, M. & Maheshwari, N. 1990. Colonic Hyperplasia And Hyperproliferation Induced By A Nutritional Stress Diet With Four Components Of Western-Style Diet. Journal Of The National Cancer Institute, 82**,** 491-496.

Nielsen, T., Sørensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M. & Sørensen, J. 2002. Antibiotic And Biosurfactant Properties Of Cyclic Lipopeptides Produced By Fluorescent Pseudomonas Spp. From The Sugar Beet Rhizosphere. Applied And Environmental Microbiology, 68**,** 3416-3423

Nicosia, R. F. & Ottinetti, A. 1990. Modulation Of Microvascular Growth And Morphogenesis By Reconstituted Basement Membrane Gel In Three-Dimensional Cultures Of Rat Aorta: A Comparative Study Of Angiogenesis In Matrigel, Collagen, Fibrin, And Plasma Clot. In Vitro Cellular & Developmental Biology, 26**,** 119-128.

Nishizuka, Y. 1984. The Role Of Protein Kinase C In Cell Surface Signal Transduction And Tumour Promotion. Nature, 308**,** 693-698.

Nitschke, M. & Costa, S. 2007. Biosurfactants In Food Industry. Trends In Food Science & Technology, 18**,** 252-259.

Nowell, P. C. 1976. The Clonal Evolution Of Tumor Cell Populations. Science, 194**,** 23-28.

Nyce, J. W., Magee, P. N., Hard, G. C. & Schwartz, A. G. 1984. Inhibition Of 1, 2-Dimethylhydrazine-Induced Colon Tumorigenesis In Balb/C Mice By Dehydroepiandrosterone. Carcinogenesis, 5**,** 57-62.

O'connell, M. J., Lavery, I., Yothers, G., Paik, S., Clark-Langone, K. M., Lopatin, M., Watson, D., Baehner, F. L., Shak, S. & Baker, J. 2010. Relationship Between Tumor Gene Expression And Recurrence In Four Independent Studies Of Patients With Stage Ii/Iii Colon Cancer Treated With Surgery Alone Or Surgery Plus Adjuvant Fluorouracil Plus Leucovorin. Journal Of Clinical Oncology, 28**,** 3937-3944.

Okada, H., Yoshida, J., Sokabe, M., Wakabayashi, T. & Hagiwara, M. 1996. Suppression Of Cd44 Expression Decreases Migration And Invasion Of Human Glioma Cells. International Journal Of Cancer, 66**,** 255-260.

Ortiz, A., Teruel, J. A., Espuny, M. J., Marqués, A., Manresa, Á. & Aranda, F. J. 2009. Interactions Of A Bacterial Biosurfactant Trehalose Lipid With Phosphatidylserine Membranes. Chemistry And Physics Of Lipids, 158**,** 46-53.

Otzen, D. E. 2016. Biosurfactants And Surfactants Interacting With Membranes And Proteins: Same But Different? Biochimica Et Biophysica Acta (Bba)-Biomembranes.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B. & Bao, J. K. 2012. Programmed Cell Death Pathways In Cancer: A Review Of Apoptosis, Autophagy And Programmed Necrosis. Cell Proliferation, 45**,** 487-498.

Owens, M. B., Hill, A. D. & Hopkins, A. M. 2013. Ductal Barriers In Mammary Epithelium. Tissue Barriers, 1**,** E25933.

Park, S. Y., Kim, J.-H., Lee, Y. J., Lee, S. J. & Kim, Y. 2013. Surfactin Suppresses Tpa-Induced Breast Cancer Cell Invasion Through The Inhibition Of Mmp-9 Expression. International Journal Of Oncology, 42**,** 287-296.

Parr, C., Hiscox, S., Nakamura, T., Matsumoto, K. & Jiang, W. G. 2000. Nk4, A New Hgf/Sf Variant, Is An Antagonist To The Influence Of Hgf/Sf On The Motility And Invasion Of Colon Cancer Cells. International Journal Of Cancer, 85**,** 563-570.

Patel, M. 2003. Surfactants Based On Renewable Raw Materials. Journal Of Industrial Ecology, 7**,** 47-62.

Patra, S. K. 2008. Dissecting Lipid Raft Facilitated Cell Signaling Pathways In Cancer. Biochimica Et Biophysica Acta (Bba)-Reviews On Cancer, 1785**,** 182-206.

Petroianu, A. 2007. Drug-Induced Splenic Enlargement. Expert Opinion On Drug Safety, 6**,** 199-206.

Pietras, K. & Östman, A. 2010. Hallmarks Of Cancer: Interactions With The Tumor Stroma. Experimental Cell Research, 316**,** 1324-1331.

Popovic, P., Popovic, V. & Baughman, J. 1981. Circadian Rhythm And 5-Fluorouracil Toxicity In C3h Mice. Progress In Clinical And Biological Research, 107**,** 185-187.

Potier, M., Chantôme, A., Joulin, V., Girault, A., Roger, S., Besson, P., Jourdan, M. L., Leguennec, J. Y., Bougnoux, P. & Vandier, C. 2011. The Sk3/Kca2. 3 Potassium Channel Is A New Cellular Target For Edelfosine. British Journal Of Pharmacology, 162**,** 464-479.

Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B. & Kinzler, K. W. 1992. Apc Mutations Occur Early During Colorectal Tumorigenesis. Nature, 359**,** 235-237.

Puppa, M. J., White, J. P., Sato, S., Cairns, M., Baynes, J. W. & Carson, J. A. 2011. Gut Barrier Dysfunction In The Apc Min/+ Mouse Model Of Colon Cancer Cachexia. Biochimica Et Biophysica Acta (Bba)-Molecular Basis Of Disease, 1812**,** 1601-1606.

Rao, V., Al-Mukhtar, A., Rayan, F., Stojkovic, S., Moore, P. & Ahmad, S. 2005. Endoscopic Laser Ablation Of Advanced Rectal Carcinoma–A Dgh Experience. Colorectal Disease, 7**,** 58-60.

Rashad, M., Nooman, M., Ali, M., Al-Kashef, A. & Mahmoud, A. 2014. Production, Characterization And Anticancer Activity Of Candida Bombicola Sophorolipids By Means Of Solid State Fermentation Of Sunflower Oil Cake And Soybean Oil. Grasas Y Aceites, 65**,** 017.

Raskin, I. & Ripoll, C. 2004. Can An Apple A Day Keep The Doctor Away? Current Pharmaceutical Design, 10**,** 3419-3429.

Rau, U., Hammen, S., Heckmann, R., Wray, V. & Lang, S. 2001. Sophorolipids: A Source For Novel Compounds. Industrial Crops And Products, 13**,** 85-92.

Reeder, C. B., Reece, D. E., Kukreti, V., Chen, C., Trudel, S., Hentz, J., Noble, B., Pirooz, N. A., Spong, J. E. & Piza, J. G. 2009. Cyclophosphamide, Bortezomib And Dexamethasone Induction For Newly Diagnosed Multiple Myeloma: High Response Rates In A Phase Ii Clinical Trial. Leukemia, 23**,** 1337-1341.

Reuter, B. K., Zhang, X.-J. & Miller, M. J. 2002. Therapeutic Utility Of Aspirin In The Apc Min/+ Murine Model Of Colon Carcinogenesis. Bmc Cancer, 2**,** 19.

Ribeiro, I. A., Faustino, C., Guerreiro, P. S., Frade, R. F., Bronze, M. R., Castro, M. F. & Ribeiro, M. H. 2015. Development Of Novel Sophorolipids With Improved Cytotoxic Activity Toward Mda-Mb-231 Breast Cancer Cells. Journal Of Molecular Recognition, 28**,** 155-165.

Ribble, D., Goldstein, N. B., Norris, D. A. & Shellman, Y. G. 2005. A Simple Technique For Quantifying Apoptosis In 96-Well Plates. Bmc Biotechnology, 5**,** 12.

Richmond, A. & Su, Y. 2008. Mouse Xenograft Models Vs Gem Models For Human Cancer Therapeutics. Disease Models And Mechanisms, 1**,** 78-82.

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. & Sporn, M. B. 1985. Type Beta Transforming Growth Factor: A Bifunctional Regulator Of Cellular Growth. Proceedings Of The National Academy Of Sciences, 82**,** 119-123.

Rodrigues, L., Banat, I. M., Teixeira, J. & Oliveira, R. 2006. Biosurfactants: Potential Applications In Medicine. Journal Of Antimicrobial Chemotherapy, 57**,** 609-618.

Rodriguez, L. G., Wu, X. & Guan, J.-L. 2005. Wound-Healing Assay. Cell Migration: Developmental Methods And Protocols**,** 23-29.

Roelants, S. L., Ciesielska, K., De Maeseneire, S. L., Moens, H., Everaert, B., Verweire, S., Denon, Q., Vanlerberghe, B., Van Bogaert, I. N. & Van Der Meeren, P. 2016. Towards The Industrialization Of New Biosurfactants: Biotechnological Opportunities For The Lactone Esterase Gene From Starmerella Bombicola. Biotechnology And Bioengineering, 113**,** 550-559.

Rodrigues, L., Banat, I. M., Teixeira, J. & Oliveira, R. 2006. Biosurfactants: Potential Applications In Medicine. Journal Of Antimicrobial Chemotherapy, 57**,** 609-618.

Roncucci, L., Stamp, D., Medline, A., Cullen, J. B. & Bruce, W. R. 1991. Identification And Quantification Of Aberrant Crypt Foci And Microadenomas In The Human Colon. Human Pathology, 22**,** 287-294

Rosenberg, D. W., Giardina, C. & Tanaka, T. 2008. Mouse Models For The Study Of Colon Carcinogenesis. Carcinogenesis, 30**,** 183-196.

Roussos, E. T., Condeelis, J. S. & Patsialou, A. 2011. Chemotaxis In Cancer. Nature Reviews Cancer, 11**,** 573-587.

Rowlatt, C., Franks, L., Sheriff, M. & Chesterman, F. 1969. Naturally Occurring Tumors And Other Lesions Of The Digestive Tract In Untreated C57bl Mice. Journal Of The National Cancer Institute, 43**,** 1353-1364.

Ruys, A. T., Alderlieste, Y. A., Gouma, D. J., Dekker, E. & Mathus–Vliegen, E. M. 2010. Jejunal Cancer In Patients With Familial Adenomatous Polyposis. Clinical Gastroenterology And Hepatology, 8**,** 731-733.

Saam, J. R. & Gordon, J. I. 1999. Inducible Gene Knockouts In The Small Intestinal And Colonic Epithelium. Journal Of Biological Chemistry, 274**,** 38071-38082.

Samad, A., Taylor, R., Marshall, T. & Chapman, M. A. 2005. A Meta-Analysis Of The Association Of Physical Activity With Reduced Risk Of Colorectal Cancer. Colorectal Disease, 7**,** 204-213

Sampson, J. R. & Jones, N. 2009. Mutyh-Associated Polyposis. Best Practice & Research Clinical Gastroenterology, 23**,** 209-218.

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V. A. & Sarubbo, L. A. 2016. Biosurfactants: Multifunctional Biomolecules Of The 21st Century. International Journal Of Molecular Sciences, 17**,** 401.

Sánchez-Tilló, E., De Barrios, O., Siles, L., Cuatrecasas, M., Castells, A. & Postigo, A. 2011. B-Catenin/Tcf4 Complex Induces The Epithelial-To-Mesenchymal Transition (Emt)-Activator Zeb1 To Regulate Tumor Invasiveness. Proceedings Of The National Academy Of Sciences, 108**,** 19204-19209.

Sánchez-Wandelmer, J., Hernández-Pinto, A. M., Cano, S., Dávalos, A., De La Peña, G., Puebla-Jiménez, L., Arilla-Ferreiro, E., Lasunción, M. A. & Busto, R. 2009. Effects Of The Antipsychotic Drug Haloperidol On The Somastostatinergic System In Sh-Sy5y Neuroblastoma Cells. Journal Of Neurochemistry, 110**,** 631-640.

Schoental, R. & Magee, P. 1962. Induction Of Squamous Carcinoma Of The Lung And Of The Stomach And Oesophagus By Diazomethane And N-Methyl-N-Nitroso-Urethane, Respectively. British Journal Of Cancer, 16**,** 92.

Savjani, K. T., Gajjar, A. K. & Savjani, J. K. 2012. Drug Solubility: Importance And Enhancement Techniques. Isrn Pharmaceutics, 2012.

Scharbert, G., Wetzel, L., Berlinger, L. & Kozek-Langenecker, S. 2011. Effect Of Anemia On Coagulation And Platelet Function: A Whole Blood In Vitro Study. Critical Care, 15**,** 1-190.

Schofield, M. H., Thavasi, T. R. & Gross, R. A. 2012. Modified Sophorolipids For The Inhibition Of Plant Pathogens. Google Patents.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. 2012. Nih Image To Imagej: 25 Years Of Image Analysis. Nature Methods, 9**,** 671.

Schröder, H. F. 1993. Surfactants: Non-Biodegradable, Significant Pollutants In Sewage Treatment Plant Effluents: Separation, Identification And Quantification By Liquid

Chromatography, Flow-Injection Analysis—Mass Spectrometry And Tandem Mass Spectrometry. Journal Of Chromatography A, 647**,** 219-234.

Schroeder, F. R. 2009. Sustainable Solutions For Consumer Products. Sustainable Solutions For Modern Economies**,** 53.

Scott, M. J. & Jones, M. N. 2000. The Biodegradation Of Surfactants In The Environment. Biochimica Et Biophysica Acta (Bba)-Biomembranes, 1508**,** 235-251.

Shah, V., Badia, D. & Ratsep, P. 2007. Sophorolipids Having Enhanced Antibacterial Activity. Antimicrobial Agents And Chemotherapy, 51**,** 397-400.

Shah, V., Doncel, G. F., Seyoum, T., Eaton, K. M., Zalenskaya, I., Hagver, R., Azim, A. & Gross, R. 2005. Sophorolipids, Microbial Glycolipids With Anti-Human Immunodeficiency Virus And Sperm-Immobilizing Activities. Antimicrobial Agents And Chemotherapy, 49**,** 4093-4100.

Shao, L., Song, X., Ma, X., Li, H. & Qu, Y. 2012. Bioactivities Of Sophorolipid With Different Structures Against Human Esophageal Cancer Cells. Journal Of Surgical Research, 173**,** 286-291.

Sheets, E. D., Holowka, D. & Baird, B. 1999. Membrane Organization In Immunoglobulin E Receptor Signaling. Current Opinion In Chemical Biology, 3**,** 95-99.

Simon, H.-U., Haj-Yehia, A. & Levi-Schaffer, F. 2000. Role Of Reactive Oxygen Species (Ros) In Apoptosis Induction. Apoptosis, 5**,** 415-418.

Simons, K. & Ehehalt, R. 2002. Cholesterol, Lipid Rafts, And Disease. The Journal Of Clinical Investigation, 110**,** 597-603.

Simons, K. & Ikonen, E. 1997. Functional Rafts In Cell Membranes. Nature, 387**,** 569.

Simons, K. & Toomre, D. 2000. Lipid Rafts And Signal Transduction. Nature Reviews Molecular Cell Biology, 1**,** 31-39.

Sivapathasekaran, C., Das, P., Mukherjee, S., Saravanakumar, J., Mandal, M. & Sen, R. 2010. Marine Bacterium Derived Lipopeptides: Characterization And Cytotoxic Activity Against Cancer Cell Lines. International Journal Of Peptide Research And Therapeutics, 16**,** 215-222.

Sleiman, J. N., Kohlhoff, S. A., Roblin, P. M., Wallner, S., Gross, R., Hammerschlag, M. R., Zenilman, M. E. & Bluth, M. H. 2009. Sophorolipids As Antibacterial Agents. Annals Of Clinical & Laboratory Science, 39**,** 60-63.

Sharma, P. M., Bowman, M., Yu, B.-F. & Sukumar, S. 1994. A Rodent Model For Wilms Tumors: Embryonal Kidney Neoplasms Induced By N-Nitroso-N'-Methylurea. Proceedings Of The National Academy Of Sciences, 91**,** 9931-9935.

Shete, A., Wadhawa, G., Banat, I. & Chopade, B. 2006. Mapping Of Patents On Bioemulsifier And Biosurfactant: A Review.

Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T. & Toyoshima, K. 1997. Rapid Colorectal Adenoma Formation Initiated By Conditional Targeting Of The Apc Gene. Science, 278**,** 120-123.

Simpson, B. 1972. The Geographic Distribution Of Carcinomas Of The Small Intestine In New Zealand Sheep. New Zealand Veterinary Journal, 20**,** 24-28.

Sleiman, J. N., Kohlhoff, S. A., Roblin, P. M., Wallner, S., Gross, R., Hammerschlag, M. R., Zenilman, M. E. & Bluth, M. H. 2009. Sophorolipids As Antibacterial Agents. Annals Of Clinical & Laboratory Science, 39**,** 60-63.

Sohrabi, B., Khani, V., Moosavi-Movahedi, A. A. & Moradi, P. 2013. Investigation Of DNA–Cationic Bolaform Surfactants Interaction With Different Spacer Length. Colloids And Surfaces B: Biointerfaces, 110**,** 29-35.

Spencer, J., Gorin, P. & Tulloch, A. 1970. Torulopsis Bombicola Sp. N. Antonie Van Leeuwenhoek, 36**,** 129-133.

Stanghellini, M. E. & Miller, R. M. 1997. Biosurfactants: Their Identity And Potential Efficacy In The Biological Control Of Zoosporic Plant Pathogens. Plant Disease, 81**,** 4-12.

Staubach, S. & Hanisch, F.-G. 2011. Lipid Rafts: Signaling And Sorting Platforms Of Cells And Their Roles In Cancer. Expert Review Of Proteomics, 8**,** 263-277.

Steger, F., Hautmann, M. G. & Kölbl, O. 2012. 5-Fu-Induced Cardiac Toxicity-An Underestimated Problem In Radiooncology? Radiation Oncology, 7**,** 212.

Steinke, V., Engel, C., Büttner, R., Schackert, H. K., Schmiegel, W. H. & Propping, P. 2013. Hereditary Nonpolyposis Colorectal Cancer (Hnpcc)/Lynch Syndrome. Dtsch Arztebl Int, 110**,** 32-8.

Stierle, A., Strobel, G. & Stierle, D. 1993. Taxol And Taxane Production By Taxomyces Andreanae, An Endophytic Fungus Of Pacific Yew. Science-New York Then Washington-
, 260**,** 214-214.

Stewart, B. & Wild, C. P. 2016. World Cancer Report 2014. World.

Stigliano, V., Sanchez-Mete, L., Martayan, A. & Anti, M. 2014. Early-Onset Colorectal Cancer: A Sporadic Or Inherited. World J Gastroenterol, 20**,** 12420-12430.

Stipcevic, T., Knight, C. P. & Kippin, T. E. 2013. Stimulation Of Adult Neural Stem Cells With A Novel Glycolipid Biosurfactant. Acta Neurologica Belgica, 113**,** 501-506

Syngal, S. 2015. Colorectal Cancer In Young Adults. Digestive Diseases And Sciences, 60**,** 722-733.

Taïeb, J., Artru, P., Paye, F., Louvet, C., Perez, N., André, T., Gayet, B., Hebbar, M.,

Goebel, F. M. & Tournigand, C. 2005. Intensive Systemic Chemotherapy Combined With Surgery For Metastatic Colorectal Cancer: Results Of A Phase Ii Study. Journal Of Clinical Oncology, 23**,** 502-509.

Tanigawa, N., Kern, D. H., Hikasa, Y. & Morton, D. L. 1982. Rapid Assay For Evaluating The Chemosensitivity Of Human Tumors In Soft Agar Culture. Cancer Research, 42**,** 2159-2164.

Tentler, J. J., Tan, A. C., Weekes, C. D., Jimeno, A., Leong, S., Pitts, T. M., Arcaroli, J. J., Messersmith, W. A. & Eckhardt, S. G. 2012. Patient-Derived Tumour Xenografts As Models For Oncology Drug Development. Nature Reviews Clinical Oncology, 9**,** 338-350.

Thompson, A. A., Dilworth, S. & Hay, R. J. 1985. Isolation And Culture Of Colonic Epithelial Cells In Serum-Free Medium. Journal Of Tissue Culture Methods, 9**,** 117-122.

Todaro, G. J., Lazar, G. K. & Green, H. 1965. The Initiation Of Cell Division In A Contact-Inhibited Mammalian Cell Line. Journal Of Cellular Physiology, 66**,** 325-333.

Tokes, A.-M., Schaff, Z., Szasz, A. M. & Kulka, J. 2013. The Distribution Of Tight Junctions And Junctional Proteins In The Human Body. Tight Junctions In Cancer Metastasis. Springer.

Tom, B. H., Rutzky, L. P., Jakstys, M. M., Oyasu, R., Kaye, C. I. & Kahan, B. D. 1976. Human Colonic Adenocarcinoma Cells. In Vitro, 12**,** 180-191

Torre, L. A., Siegel, R. L., Ward, E. M. & Jemal, A. 2016. Global Cancer Incidence And Mortality Rates And Trends—An Update. Cancer Epidemiology Biomarkers & Prevention, 25**,** 16-27.

Tucker, J. M., Davis, C., Kitchens, M. E., Bunni, M. A., Priest, D. G., Spencer, H. T. & Berger, F. G. 2002. Response To 5-Fluorouracil Chemotherapy Is Modified By Dietary Folic Acid Deficiency In Apcmin/+ Mice. Cancer Letters, 187**,** 153-162.

Turrens, J. F. 2003. Mitochondrial Formation Of Reactive Oxygen Species. The Journal Of Physiology, 552**,** 335-344.

Van Bogaert, I. N., Saerens, K., De Muynck, C., Develter, D., Soetaert, W. & Vandamme, E. J. 2007. Microbial Production And Application Of Sophorolipids. pplied Microbiology And Biotechnology, 76**,** 23-34.

Vali, S. R., Chakrabarti, P. P. & Thengumpillil, N. B. K. 2005. Process For The Isolation Of Glycolipids. Google Patents.

Van Bogaert, I. N., Buyst, D., Martins, J. C., Roelants, S. L. & Soetaert, W. K. 2016. Synthesis Of Bolaform Biosurfactants By An Engineered Starmerella Bombicola Yeast. Biotechnology And Bioengineering, 113**,** 2644-2651.

Van Bogaert, I. N., Saerens, K., De Muynck, C., Develter, D., Soetaert, W. & Vandamme, E. J. 2007. Microbial Production And Application Of Sophorolipids. Applied Microbiology And Biotechnology, 76**,** 23-34.

Van Bogaert, I. N., Zhang, J. & Soetaert, W. 2011. Microbial Synthesis Of Sophorolipids. Process Biochemistry, 46**,** 821-833.

Vargas, A. J. & Thompson, P. A. 2012. Diet And Nutrient Factors In Colorectal Cancer Risk. Nutrition In Clinical Practice, 27**,** 613-623.

Vemula, V. R., Lagishetty, V. & Lingala, S. 2010. Solubility Enhancement Techniques. International Journal Of Pharmaceutical Sciences Review And Research, 5**,** 41-51.

Van Zeeland, A. A. 1996. Molecular Dosimetry Of Chemical Mutagens. Relationship Between Dna Adduct Formation And Genetic Changes Analyzed At The Molecular Level. Mutation Research/Fundamental And Molecular Mechanisms Of Mutagenesis, 353**,** 123-150.

Vasen, H. F., Möslein, G., Alonso, A., Bernstein, I., Bertario, L., Blanco, I., Burn, J., Capella, G., Engel, C. & Frayling, I. 2007. Guidelines For The Clinical Management Of Lynch Syndrome (Hereditary Non-Polyposis Cancer). Journal Of Medical Genetics, 44**,** 353-362.

Vollenbroich, D., Özel, M., Vater, J., Kamp, R. M. & Pauli, G. 1997. Mechanism Of Inactivation Of Enveloped Viruses By The Biosurfactant Surfactin Frombacillus Subtilis. Biologicals, 25**,** 289-297.

Wang, J. & Yi, J. 2008. Cancer Cell Killing Via Ros: To Increase Or Decrease, That Is The Question. Cancer Biology & Therapy, 7**,** 1875-1884.

Weaver, B. A. 2014. How Taxol/Paclitaxel Kills Cancer Cells. Molecular Biology Of The Cell, 25**,** 2677-2681.

Wilding, J. L. & Bodmer, W. F. 2014. Cancer Cell Lines For Drug Discovery And Development. Cancer Research, 74**,** 2377-2384.

Weindruch, R., Walford, R. L., Fligiel, S. & Guthrie, D. 1986. The Retardation Of Aging In Mice By Dietary Restriction: Longevity, Cancer, Immunity And Lifetime Energy Intake. J Nutr, 116**,** 641-54.

Wood, P. A., Yang, X., Taber, A., Oh, E.-Y., Ansell, C., Ayers, S. E., Al-Assaad, Z., Carnevale, K., Berger, F. G. & Peña, M. M. O. 2008. Period 2 Mutation Accelerates Apcmin/+ Tumorigenesis. Molecular Cancer Research, 6**,** 1786-1793.

Worthley, D. L. & Leggett, B. A. 2010. Colorectal Cancer: Molecular Features And Clinical Opportunities. Clin Biochem Rev, 31**,** 31-8.

Wirtzfeld, D. A., Petrelli, N. J. & Rodriguez-Bigas, M. A. 2001. Hamartomatous Polyposis Syndromes: Molecular Genetics, Neoplastic Risk, And Surveillance Recommendations. Annals Of Surgical Oncology, 8**,** 319-327.

Wu, Q. & Wu, S. 2012. Lipid Rafts Association And Anti-Apoptotic Function Of Prohibitin In Ultraviolet B Light-Irradiated Hacat Keratinocytes. Experimental Dermatology, 21**,** 640-642.

Yekkala, K. & Baudino, T. A. 2007. Inhibition Of Intestinal Polyposis With Reduced Angiogenesis In Apcmin/+ Mice Due To Decreases In C-Myc Expression. Molecular Cancer Research, 5**,** 1296-1303.

Yoo, D.-S., Lee, B.-S. & Kim, E.-K. 2005. Characteristics Of Microbial Biosurfactant As An Antifungal Agent Against Plant Pathogenic Fungus. Journal Of Microbiology And Biotechnology, 15**,** 1164-1169.

You, S., Ohmori, M., Peña, M. M. O., Nassri, B., Quiton, J., Al-Assad, Z. A., Liu, L., Wood, P. A., Berger, S. H. & Liu, Z. 2006. Developmental Abnormalities In Multiple Proliferative Tissues Of Apcmin/+ Mice. International Journal Of Experimental Pathology, 87**,** 227-236.

Young, C. B. F. & Coons, K. 1945. Surface Active Agents, Theoretical Aspects And Applications. Surface Active Agents, Theoretical Aspects And Applications.

Zana, R. & Xia, J. 2003. Gemini Surfactants: Synthesis, Interfacial And Solution-Phase Behavior, And Applications, Crc Press.

Zbuk, K. M. & Eng, C. 2007. Hamartomatous Polyposis Syndromes. Nature Clinical Practice Gastroenterology & Hepatology, 4**,** 492-502.

Zhang, A. Y., Yi, F., Zhang, G., Gulbins, E. & Li, P.-L. 2006. Lipid Raft Clustering And Redox Signaling Platform Formation In Coronary Arterial Endothelial Cells. Hypertension, 47**,** 74-80.

Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R. & Freeman, M. R. 2005. Cholesterol Targeting Alters Lipid Raft Composition And Cell Survival In Prostate Cancer Cells And Xenografts. The Journal Of Clinical Investigation, 115**,** 959-968.

Zweibaum, A., Laburthe, M., Grasset, E. & Louvard, D. 2011. Use Of Cultured Cell Lines In Studies Of Intestinal Cell Differentiation And Function. Comprehensive Physiology.

Appendix 1

Supplementary data

Table A1.1 – Data sheet to score animal behaviour during treatment

Cage number:
Study number:

Dead
In nest
Weight
Fur appearance • Normal • Pilo-erection • Bald • other
Walking • Normal • Circles • Unable to stand on 4 legs • other
Ears open
Eyes open
Posture • Normal • Huddled

• Other
Reaction to cage opening • Active • Hyperactive • No response
Reaction of handling. • None • Irritation/biting • Fear (faeces/urine)
Cleaning aggression (+/-)
Whister chewing (+/-)
Comment

Figure A1.2 - The structure of BfSL and their effects on cell viability

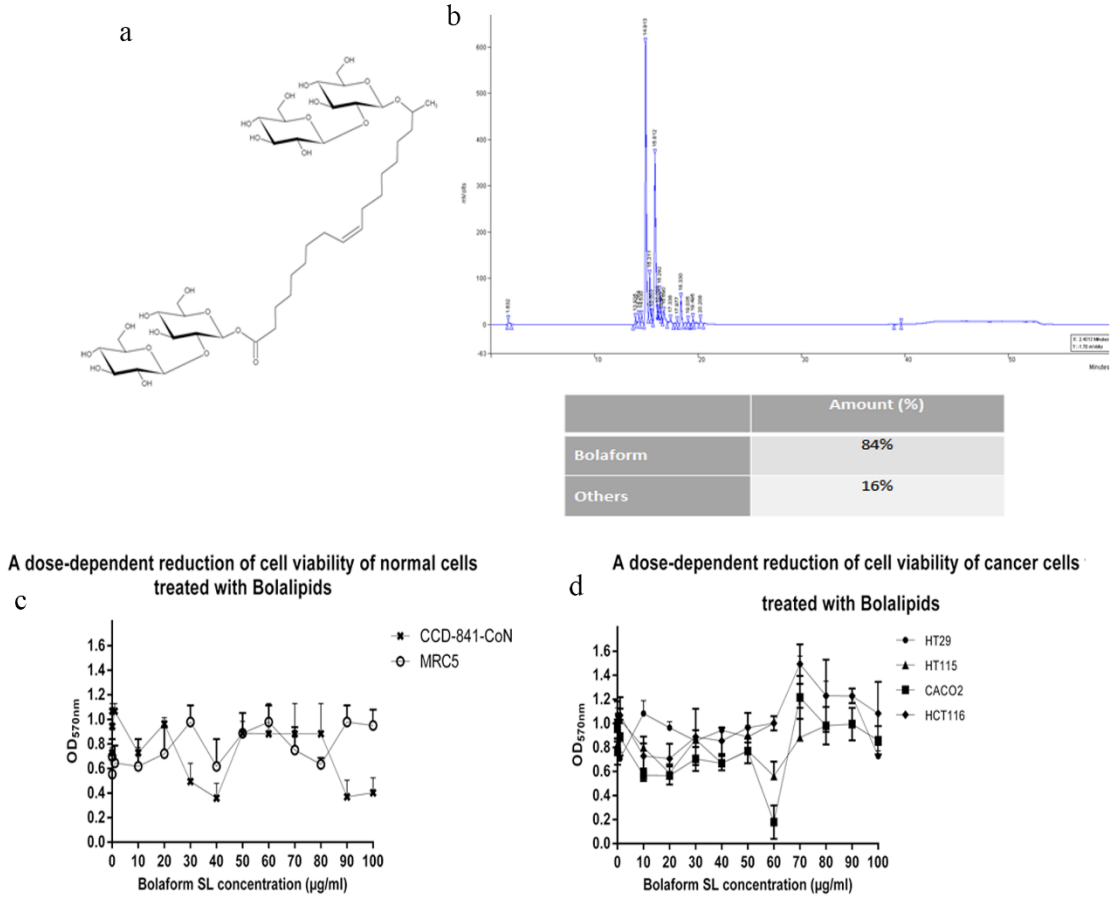
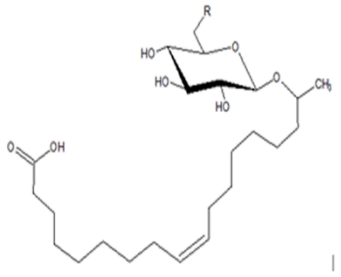
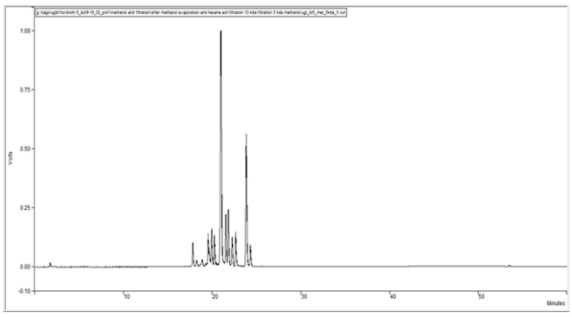


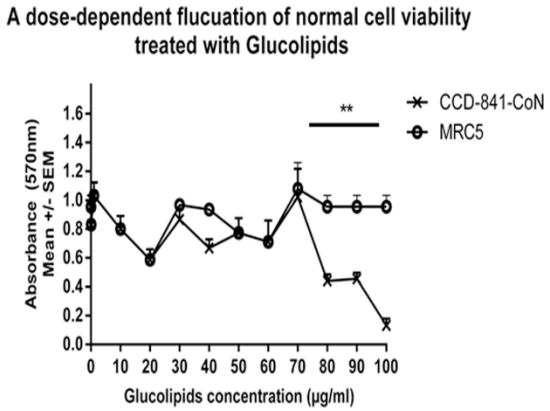
Fig A1.2 – Bolaform SL. a) the structure of BfSL consists of an open structure with two sophorose heads attached to either end of a lipid. b) HPLC-ELSD analysis of the BfSL precipitate from the bioreactor. (a) The large peak corresponds to diacetyl BfSL. HPLC-ELSD data shows this compound is made up of 84% BfSL and 16% unknown. c+d – Cell viability of normal CCD-841-CoN and MRC5 (c) and CRC cells, HT29, HT115, Caco2 and HCT116 treated with BfSL in vitro (d). Following 24h of treatment with BfSL we observed a fluctuation in O.D.570 values at doses between 10–100µg/ml in the control cell lines CCD-841-CoN and MRC5. (d) A fluctuation in O.D.570 values were also observed at doses between 10–100µg/ml in all CRC cell lines. Graphs show a representative data set from three independent experimental replicates. Values indicate mean ± SEM (n = 3).

Figure A1.3 - The structure of Glucolipids and their effects on cell viability





	Amount (%)
GL	74%
Others	26%



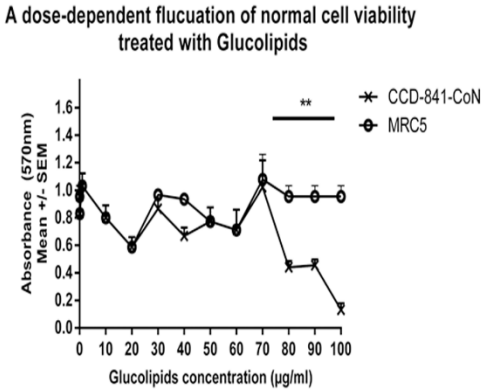
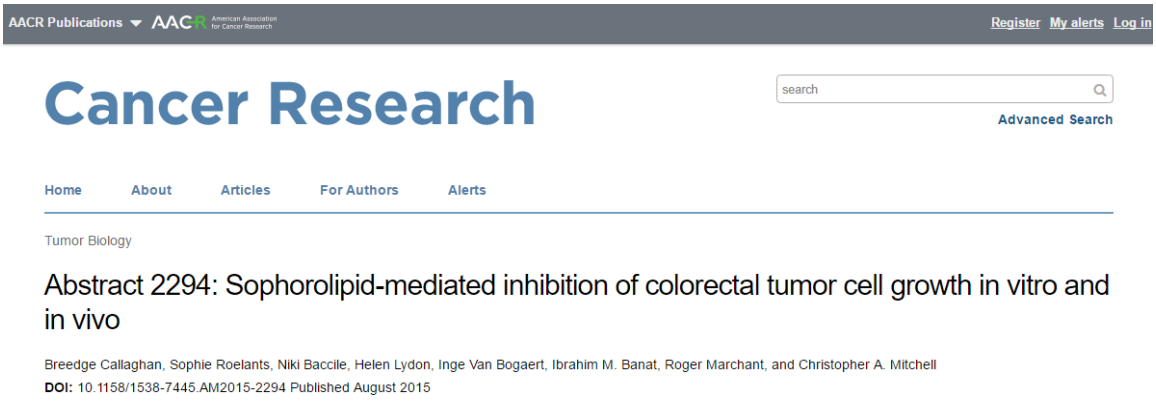


Fig A1.3 – Glucolipids (GL). a) the structure of GL consists of an open structure with a glucose head attached to a lipid. b) HPLC-ELSD analysis of the GL precipitate from the bioreactor (a). HPLC-ELSD data shows this compound is made up of 74% BfSL and 26% unknown. c+d – Cell viability of normal CCD-841-CoN and MRC5 (c) and CRC cells, HT29, HT115, Caco2 and HCT116 treated with GL in vitro (d). Following 24h of treatment with GL, a fluctuation in O.D.570 values at doses between 10–100µg/ml in the control cell lines CCD-841-CoN and MRC5 was observed. A steady decrease in viability of CCD-841-CoN was observed from 70µg/ml (**p<0.001) (d) A fluctuation in O.D.570 values were also observed at doses between 10–100µg/ml in all CRC cell lines treated with GL. Graphs show a representative data set from three independent experimental replicates. Values indicate mean ± SEM (n = 3). Statistical significance was assessed one-way ANOVA (*p <0.01; **p < 0.001; ***p < 0.0001)

Appendix 2

Published Conference Abstracts



Background: Sophorolipids (SL), are amphiphilic biosurfactant molecules which consist of a sophorose molecule with 2 variable chain length (C10 - C22) They contain double bonds at the 3" 4" positions and fatty acids at the 1" positions. SL exist in either a lactonic (SL^L; closed ring) or acidic (SL^A; open ring) forms. SL is produced in crude mixtures by the yeast *Candidia bombicola* in economically viable amounts, with variable levels of anti-proliferative activity on tumour cell lines in vitro. As biosurfactants are well tolerated in the GI tract and currently used in a variety of food products, we tested the hypotheses that purified forms of either SL^L or SL^A have differential effects on colo-rectal tumour versus "normal" cells as well as in a well-established model of pre-cancerous lesions; viz the $Apc^{min+/-}$ mouse.

Methodology: The colo-rectal cancer cell lines HT29, HT115, HCT116, Caco-2 in addition to CCD-841 colonic epithelium and MRC5 lung fibroblasts were exposed to 10 - 100 µg/ml of either SL^L or SL^A (96% or 94%) pure respectively by HPLC/MS analysis) for 24 hours following serum starvation and an MTT assay performed. The mechanism of cell death in cell lines was assessed by acridine orange/ethidium bromide staining followed by microscopic examination. Five-week old Apc^{min+} mice or wild-type littermate mice were treated orally with 50mg/kg of either SL^L or SL^A, or vehicle-only control every other day for 14 weeks. Weights, water and food consumption were measured on a daily basis. On completion of the experiment, mice were euthanized, the

digestive tract was excised, washed and fixed with 10% BFS. Polyp size, number and location were recorded and samples were blocked for paraffin embedding, sectioning and H&E/immunofluorescence staining and analysis.

Results and Discussion: In vitro, SL^L caused a decrease in colorectal cancer cell viability at >70μg/ml. However, it also caused an unfavourable effect on the colonic epithelium and lung fibroblast cell lines by reducing cell viability at a lower concentration (<10μg/ml). In-vivo, SL^L treatment exacerbated the growth of polyps along the digestive tract of Apc^{min+} mice. SL^L treatment resulted in an increase in polyp number and size compared to vehicle treated $Apc^{min+/-}$ mice. SL^L treated mice presented with reduced haematocrits (28.5% vs 34.5% $p<0.03$) and increased spleen weights (0.56g vs 0.70g $p<0.01$) compared to vehicle treated control Apc^{min+} mice.

SL^A induced a dose dependent decrease in cell viability of colo-rectal cancer cell lines (20μg/ml) without affecting the viability of the colonic epithelial and lung cell lines. SL^A selectively induced cell death in the colo-rectal cancer cells by means of necrosis. However, the effects of SL^A in vivo are yet to be established.

Contrary to current literature, purified SL^A appears to have an advantage over the SL^L form in vitro. To date, this is the first study investigating anti-cancer effects of sophorolipids in vivo.

Appendix 3

Publications

PLOS ONE

RESEARCH ARTICLE

Lactonic Sophorolipids Increase Tumor Burden in Apc$^{min+/-}$ Mice

Breedge Callaghan[1], Helen Lydon[1], Sophie L. K. W. Roelants[2], Inge N. A. Van Bogaert[2], Roger Marchant[1], Ibrahim M. Banat[1], Christopher A. Mitchell[1]*

1 Biomedical Sciences Research Institute, Centre for Molecular Biosciences, Ulster University, Coleraine, BT52 1SA, United Kingdom, **2** Centre for Industrial Biotechnology and Biocatalysis (InBio.be), Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000, Ghent, Belgium

* ca.mitchell@ulster.ac.uk

Abstract

Sophorolipids (SL) are amphiphilic biosurfactant molecules consisting of a disaccharide sophorose with one fatty acid at the C1 position and optional acetylation at the C6'and C6" positions. They exist in a closed ring lactonic (LSL) or open acidic (ASL) structure Sophorolipids are produced in crude mixtures in economically viable amounts by the yeast *Starmerella bombicola* and used in a variety of consumer products. Varying levels of antiproliferative and anti-cancer activity of crude sophorolipid mixtures are described in a number of tumor cell lines *in vitro*. However, significant inter-study variation exists in the composition of sophorolipid species as well as other biologically active compounds in these mixtures, which makes interpretation of *in vitro* and *in vivo* studies difficult. We produced a 96% pure C18:1 lactonic sophorolipid that dose-dependently reduces the viability of colorectal cancer, as well as normal human colonic and lung cell lines *in vitro*. Oral administration of vehicle-only; or lactonic sophorolipids (50 mg/kg for 70 days), to Apc$^{min+/-}$ mice resulted in an increase in the number (55.5 ± 3.3 *vs* 70.50 ± 7.8: $p < 0.05$) and size (modal size 2mm vs 4mm) of intestinal polyps. Lactonic administration resulted in a systematic effect via reduced hematocrit (49.5 ± 1.0 vs 28.2 ± 2.0 *vs*: $p<0.03$) and splenomegaly (0.56 ± 0.03g vs 0.71 ± 0.04g; $p<0.01$) confirming exacerbation of disease progression in this model.

OPEN ACCESS

Citation: Callaghan B, Lydon H, Roelants SLKW, Van Bogaert INA, Marchant R, Banat IM, et al. (2016) Lactonic Sophorolipids Increase Tumor Burden in Apc$^{min+/-}$ Mice. PLoS ONE 11(6): e0156845. doi:10.1371/journal.pone.0156845

Editor: Chunming Liu, University of Kentucky, UNITED STATES

Received: February 11, 2016

Accepted: May 20, 2016

Published: June 6, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This research was funded by the European FP7 Project Biosurfing, Grant number 289219. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Biosurfactants are produced by a variety of microorganisms as secondary metabolites, forming emulsions that reduce both interfacial and surface tension [1]. Due to their increased biodegradability, low toxicity and ability to exert an effect at extreme temperatures and pH levels [2], they prove versatile for a wide range of biomedical and industrial applications [3]. Currently, a range of microbial biosurfactants are used in cleaning supplies [4], pesticides [5], textiles [6] and cosmetics [7] while petroleum derived surfactants are still used in food products [8] and over the counter creams [9]. Microbial biosurfactants are a diverse group of surface- active compounds classified by their chemical structure, weight and microbial origin [10]. Some well-

known glycolipid biosurfactants include sophorolipids (SL), mannosylerythritols, trehalolipids and rhamnolipids [11].

The SL species we describe in this study are produced by different types of yeast such as *Starmerella bombicola*, *Candida bastistaeic*, *C. floricola and C. apicola* [12]. In these organisms the SL species are composed of a hydrophobic fatty acid tail and a hydrophilic carbohydrate head composed of a disaccharide sophorose linked by a β-1, 2 bond which is optionally acetylated on the 6' and/or 6" position. The structure of SLs is dependent on a terminal or sub-terminal hydroxylated fatty acid, which is linked β-glycosidically to the sophorose. The fatty acids' carboxylic end can be free, forming the acidic structure [13] or can be esterified at the 4" position giving rise to the lactonic ring structure (Fig 1).

A wide range of bioactivities for SL have been documented; including antimicrobial activity *via* membrane destabilization and increased permeabilization [14] and anti-inflammatory effects through the reduction of cytokine release and initiation of a macrophage response [15]. Several studies indicate that LSL [16–18] show greater potentials as anti-tumor, anti-microbial, anti- fungal and spermicide agents, while ASL are more suited as moisturizing, solubilizing, cleaning and emulsifying agents. The purity and composition of SL used in bioassays is highly variable, with most studies not disclosing the molecular species or their relative abundances within the mixtures [19–21]. Additionally, SL analogs and their derivatives can reduce the efficacy of crude preparations [16, 19] and they are known to exhibit varying potencies and toxicities depending on their manufacturing methods [22]. LSL normally make up the highest proportion of crude preparations of SL (characteristically 70–85% [23]), with the remainder comprised of varying amounts of ASL and other derivatives; the aforementioned considerations underline the need to use purified and characterized (ASL or LSL) forms when assessing or comparing bioactivities. In order to minimize batch to batch variation and reduce specific congeners (2.5% ASL and <1% free fatty acids as a proportion of dry weight) we used a tightly-controlled batch fermentation method in order to produce a stock of highly pure and well characterized LSL that was used in all our *in vitro* and *in vivo* studies.

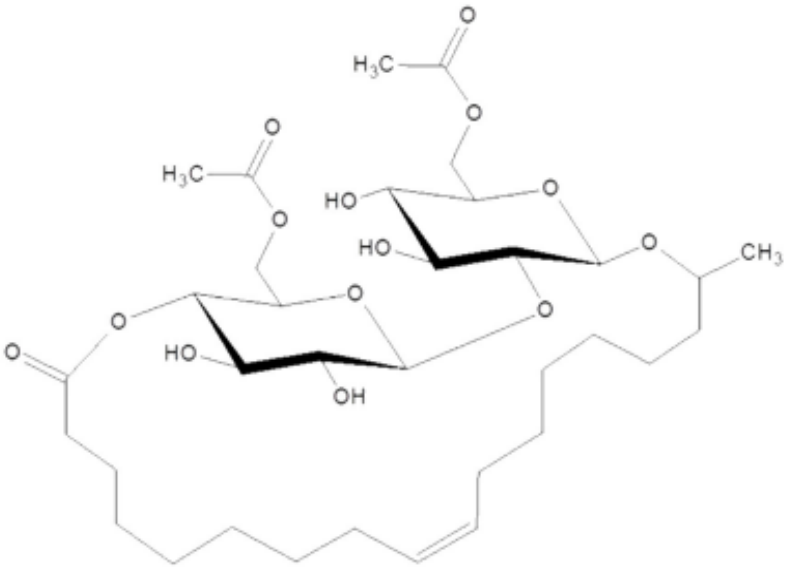


Fig 1. Lactonic Sophorolipid. The structure of C18:1 diacetylated LSL.

doi:10.1371/journal.pone.0156845.g001

The *in vitro* activities of SL has been reviewed [12], with enriched preparations showing potent cytotoxicity against human liver (HT402), lung (A549) and leukemic (HL60 & K562) cells [24]. Cruder preparations of SL are reported to show dose-dependent, anti-proliferative and pro- apoptotic activity against pancreatic cancer (H7402, HPAC) [19] and esophageal cancer (KYSE450, KYSE109) cell lines [17]. Conclusions on the specificity of such diverse preparations of SL to transformed cells is complicated by the inappropriate use of controls—many studies lack the use of appropriate primary or non-transformed cells such as non-adherent peripheral blood mononuclear cells (PBMCs) [25].

Despite a number of studies showing that SL preparations have anti-proliferative effects on tumor cell lines, to our knowledge the anti-tumor effects of these compounds *in vivo* has not been reported. However, SL mixtures have been shown to reduce mortality and regulate nitric oxide production in a rat model of peritoneal sepsis [14], as well as reducing IgE production [26] following nebulizer administration in a mouse model of asthma [27]. These pre-clinical studies are consistent with the proposal that parenteral administration of relatively low doses of SL is safe and non-toxic in-*vivo*.

We hypothesized that purified forms of LSL would specifically inhibit colorectal tumor cell growth both *in vitro* and *in vivo*. Therefore, we purified and characterized a LSL preparation produced by *Starmerella bombicola* and assessed its' effects on five unrelated colorectal cancer cell lines: HT29, HT115, HCT116, Caco-2 and LS180, in addition to two non-transformed lines: normal human colonic epithelium CCD-841 CoN and lung fibroblast MRC5. In addition, we administered 50mg/kg of LSL orally for 70 days to Apc$^{min+/-}$ mice (a well-established model of colorectal neoplasia) [28, 29], to determine its' ability to inhibit tumor growth *in vivo*.

Materials and Methods

Sophorolipid production and purification

A lactonesterase overexpressing strain of *Starmerella bombicola* (oe *sble*) as described by Roelants *et al.* [30] was used for the production of 96% pure lactonic diacetylated SL. HPLC- ELSD and LC-MS analysis was performed as described by Roelants *et al.* [30].

Cell culture

The colorectal cancer cell lines HT29 (ATCC® HTB-38), HT115 (ECACC-cultures 85061104) HCT116 (ATCC® CCL-247), LS180 (ATCC® CL-187), Caco-2 (ATCC® HTB-37), normal colonic epithelium CCD-841-CoN (ATCC® CRL-1790) and lung fibroblasts MRC5 (ATCC® CCL-171) were maintained in DMEM media supplemented with 10% fetal bovine serum (Gibco Invitrogen; Paisley, UK). All cultures were maintained at 37°C and at 5% CO2.

MTT assay

A total of 5x10^4 cells per well were seeded (96 well plate: Nunc Thermos scientific, UK) and allowed to attach overnight before being serum starved for 24h. Various concentrations of LSL (0.001 μg/ml– 100 μg/ml) were added to the cultures and incubated for another 24h. Subsequently, 10μl of a 25mg/ml solution of MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide; (Sigma-Aldrich Company Ltd, Dorset, UK) was added to each well and the plate was further incubated for 1h at 37°C. The formazan crystals were solubilized with 100μl of DMSO (Sigma-Aldrich Company Ltd, Dorset, UK) and the absorbance at 570nm was read on a spectrophotometer plate-reader (BMG-LABTECH, Omega, Aylesbury, Bucks UK). Each experiment was repeated 3 times with 6 internal repeats per group.

Acridine orange/ethidium bromide staining and quantitation

To determine the number of necrotic or apoptotic cells induced by addition of LSL, cells were stained *in situ* with 10mg/ml acridine orange (Sigma-Aldrich Company Ltd, Dorset, UK) and 1mg/ml ethidium bromide (Sigma-Aldrich Company Ltd, Dorset, UK) and morphological changes were assessed by fluorescence microscopy [31]. For assessment of apoptosis, a total of $3x10^4$ cells were seeded onto a 10mm coverslip (Agar Scientific; Stansted, Essex, UK) and incubated overnight to form a confluent monolayer. Following serum starvation for 24h, LSL (20μg/ml or 70μg/ml) or 5μM of etoposide (control) (Sigma-Aldrich Company Ltd, Dorset, UK) was added and the cells incubated for a further 24h. To determine the number of live cells remaining on the coverslip the samples were washed three times with ice-cold phosphate buffered saline (PBS; pH7.4, Oxoid: UK) 3 times, followed by incubation with a solution of 10μl of 1:1 acridine orange/ethidium bromide for 5 minutes and then the cells were washed 3x with ice-cold PBS and subsequent imaging with a Zeiss florescence microscope (Axio Scope 1, Zeiss, Germany) at a range of objective magnifications. The operator was blinded to the experimental groups and random fields were selected (40X objective). A total of 300 attached cells per coverslip were morphologically identified and counted as being either necrotic (red/orange nuclei), apoptotic (green condensed or fragmented nuclei) green or live (green non-condensed ovoid or rounded nuclei).

Animal model

All animal procedures were approved by the animal care and ethics committee (Ulster University) and national (UK Home Office) ethical guidelines, and also carried out in accordance with both local animal care committee (Ulster University) and national (UK Home Office) guidelines by licensed personnel [32]. Apc$^{min+/-}$ male and wild type (*wt*) female mice were housed together for breeding purposes and subjected to a 12/12 light cycle. Food and water were available *ad libitum* and weighed on a weekly basis to evaluate consumption. Animal husbandry was carried out bi-weekly. Mice were monitored on a daily basis looking at grooming, behavior, activity levels, food and water in-take and general well-being. Mice deemed un-well were immediately removed from the study and euthanized. Body weights were monitored bi-weekly. A cut-off point of 10% loss of body weight was applied and mice reaching this threshold were euthanized immediately by CO2.

Genotyping

Ear punch samples were obtained from the 21 day old progeny of Apc$^{min+/-}$/*wt* crosses for the purpose of genotyping, using primers specific for the APC mutation. DNA was isolated from ear samples by first solubilizing them in an alkaline lysis reagent (25mM NaOH, 0.2MmM disodium EDTA; (Sigma-Aldrich Company Ltd, Dorset, UK) at 95°C for 40 minutes, allowing them to cool and neutralizing in Tris-HCl (40mM; (Sigma-Aldrich Company Ltd, Dorset, UK).

Digested samples were mixed with PCR master mix (Qiagen Company Ltd, Manchester, UK), *taq* (Qiagen Company Ltd, Manchester UK), nuclease free water and Apc^{min+} specific primers (100μm each) (Forward: TCT CGT TCT GAG AAA GAC AGA AGC T, Reverse: TGA TAC TTC TTC CAA AGC TTT GGC TAT; Invitrogen Company Ltd, Paisley, UK). Samples were placed in a thermocycler (Techne TC-5000 Gradient Thermocycler, Hanwell, London UK) and a PCR reaction performed under the following conditions: 94°C, 2min; (94°C, 1min; 60°C, 1min; 72°C, 1min) for 30 cycles followed by 72°C for 2 minutes. PCR products were subjected to *Hind*III digestion (Invitrogen, Paisley, UK) for 1h at 37°C followed by a 20 minute denaturing step at 65°C. Digests were run on a 4% agarose/TBE-buffered gel (Sigma-Aldrich, Dorset,

UK) for 40 minutes. The presence of a single band at 111bp indicated a *wt* mouse, while an additional band at 123bp indicated a heterozygous Apc$^{min+/-}$ mouse.

Sophorolipid dosing

At five weeks of age, equal numbers of *wt* and Apc$^{min+/-}$ males and females were placed into experimental groups. Mice were treated orally (*via* a sterile p20 pipette tip) every other day with either vehicle-only or a solution containing 50mg/kg (body weight) of LSL suspended in 0.1% ethanol/ 10% sucrose for 70 days.

Tissue collection and assessment

Mice were euthanized with an overdose of general anesthetic, blood immediately collected by cardiac puncture into EDTA tubes (Aquilant Scientific, Down, NI) and hematocrit determined (Cole-parmer, Trickenham,UK). Intestinal tract, colon spleen, heart, liver, kidneys and lungs were carefully removed, weighed and then fixed in 10% buffered formal saline (pH7.4). The intestinal tracts were divided into 3 sections according to the description of Casteleyn *et al.* (2010) [33]. After identification of the specific intestinal regions, samples were bisected longitudinally and the number of polyps was recorded as well as their diameters measured with calipers. The specimens were then cut into ~2cm strips and placed in cassettes prior to standard tissue processing and wax embedding. To assess qualitative histopathological changes in the intestines and spleen, tissues were cut into 5μm sections using a microtome (Shandon; Cheshire, UK) placed on glass slides, cleared with xylene, dehydrated in descending grades of ethanol, stained with Mayer's haematoxylin and eosin stain (Sigma-Aldrich, Dorset, UK) and examined with a Zeiss light microscope (Axio Scope 1, Zeiss, Germany) at a range of objective magnifications.

Statistical analysis

Statistical analysis of *in vitro* data was determined using either one-way ANOVA or student's t- test using GraphPad Prism (GraphPad software, San Diego, USA). All comparisons between *in vivo* groups were assessed using a students' t-test. A value of $p < 0.05$ was defined statistically significant.

Results

Production and purification of LSL

To produce the C18:1 lactonic diacetylated SL used for our *in vitro* and *in vivo* studies (Fig 1), we employed the *S. bombicola* oe *sble* strain and a bioreactor experiment similar to one previously described [30]; however, instead of rapeseed oil, oleic acid was used as the hydrophobic carbon source. This resulted in a very uniform SL product (Fig 2a) containing 99% SL (97.3% C18:1 SL [Mw = 688], 1.3% C18:2 SL and 0.4% C18:0 SL).

The purification process was previously described [30] and consists of several washing steps with demineralized water of the spontaneously precipitated LSL product from the bioreactor. The washing steps remove hydrophilic impurities such as salts, sugars and proteins from the water-insoluble LSL. Hydrophobic impurities did not have to be removed, because the feeding rate of oleic acid was adjusted to its consumption rate, to avoid accumulation and the presence of an excess of substrate at the end of the fermentation. This is a large advantage, as solvent extractions to remove oil/fatty acids can be avoided. A final crystallization step of the LSL at 4°C, followed by lyophilisation gave rise to a dry and stable white powder. The final composition of the sample was analyzed using HPLC-ELSD and determination of possible congeners

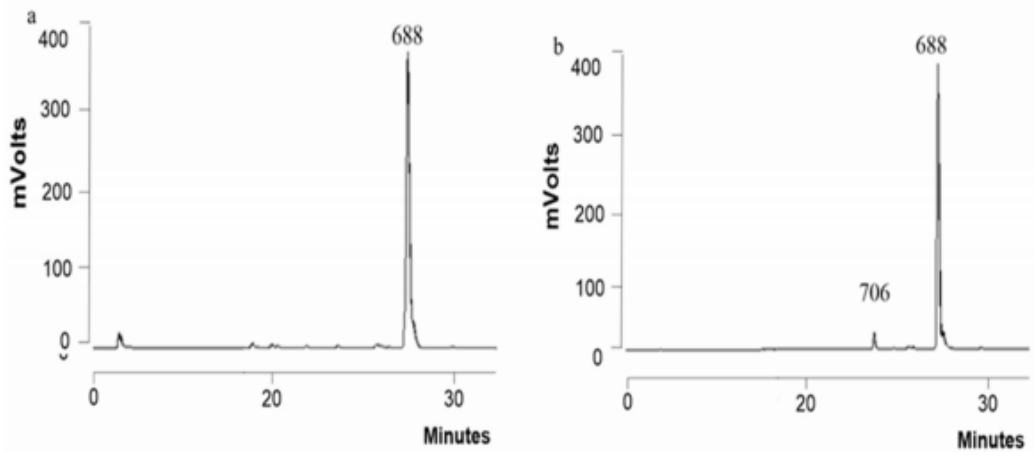


Fig 2. Purification of LSL produced by S. bombicola. HPLC-ELSD and LC-MS analysis of the LSL precipitate from the bioreactor. (a) The MW 688 g/mol corresponds to diacetyl C18:1 LSL. (b) HPLC-ELSD analysis following purification of the LSL precipitate. The MW 706 g/mol = diacetyl C18:1 ASL, which is the result of minor hydrolysis of the MW 688 compound.

doi:10.1371/journal.pone.0156845.g002

(e.g. salts, sugars proteins and oleic acid) was performed. The final purity of the sample was >99.5% SL and the composition in terms of SL was determined to be 96% diacetylated C18:1 LSL (Mw = 688; Fig 2b), 3.8% diacetylated C18:1 ASL (Mw = 706; Fig 2b) and minor impurities consisting of 0.04% free fatty acids/oil, 0.001% glucose, 0.004% glycerol and total nitrogen of 0.14%. The ASL was generated by hydrolysis of the LSL in the first step of the purification, which consisted of heating (65°C) of the culture broth, to melt and subsequently precipitate the SL product. This process was later optimized in order to avoid this unwanted hydrolysis [30].

LSL have a differential effect on colorectal cell viability

In culture, LSL concentrations above 20μg/ml resulted in reduced viability of both colonic epithelial (CCD-841-CoN) and lung fibroblast (MRC5) cell lines (Fig 3a; $p<0.0001$), in addition to Caco2, HCT116 and LS180 colorectal tumor cell lines (Fig 3b; $p < 0.05$). HT29 cells initially appear to increase in viability at doses between 20–40μg/ml; however this phenomenon is not statistically significant from vehicle-only control values ($p > 0.05$). In both HT29 and HT115 colorectal cancer cell lines a significant decrease in viability was observed at doses exceeding 70μg/ml ($p < 0.001$; Fig 3b). Microscopic examination of confluent cultures of CCD- 841-CoN cells revealed a bipolar morphology following exposure to vehicle, whereas at doses of 40 and70 μg/ml there were large areas devoid of cells, with remaining adherent cells displaying a shrunken and rounded phenotype (Fig 3c: top). In vehicle-treated cultures, HT29 cells display densely packed, cobblestone-like morphology (Fig 3c: bottom) and there was no obvious change in phenotype at a dose of 40 μg/ml LSL. In HT29 cells exposed to 70μg/ml LSL, the confluent monolayer was disturbed and there were clear signs of cell rounding and cell-free areas indicative of detachment (Fig 3c: bottom). Detached cells were isolated from the supernatant of wells treated with 0, 50 and 100μg/ml LSL to determine if they were alive or dead using propidium iodide and Syto 9 staining. At 50 and 10μg/ml, all cells found in the normal CCD-841-CoN supernatant were dead (S1A Fig $p<0.001$). In the cancer cell lines, 4% of cells were

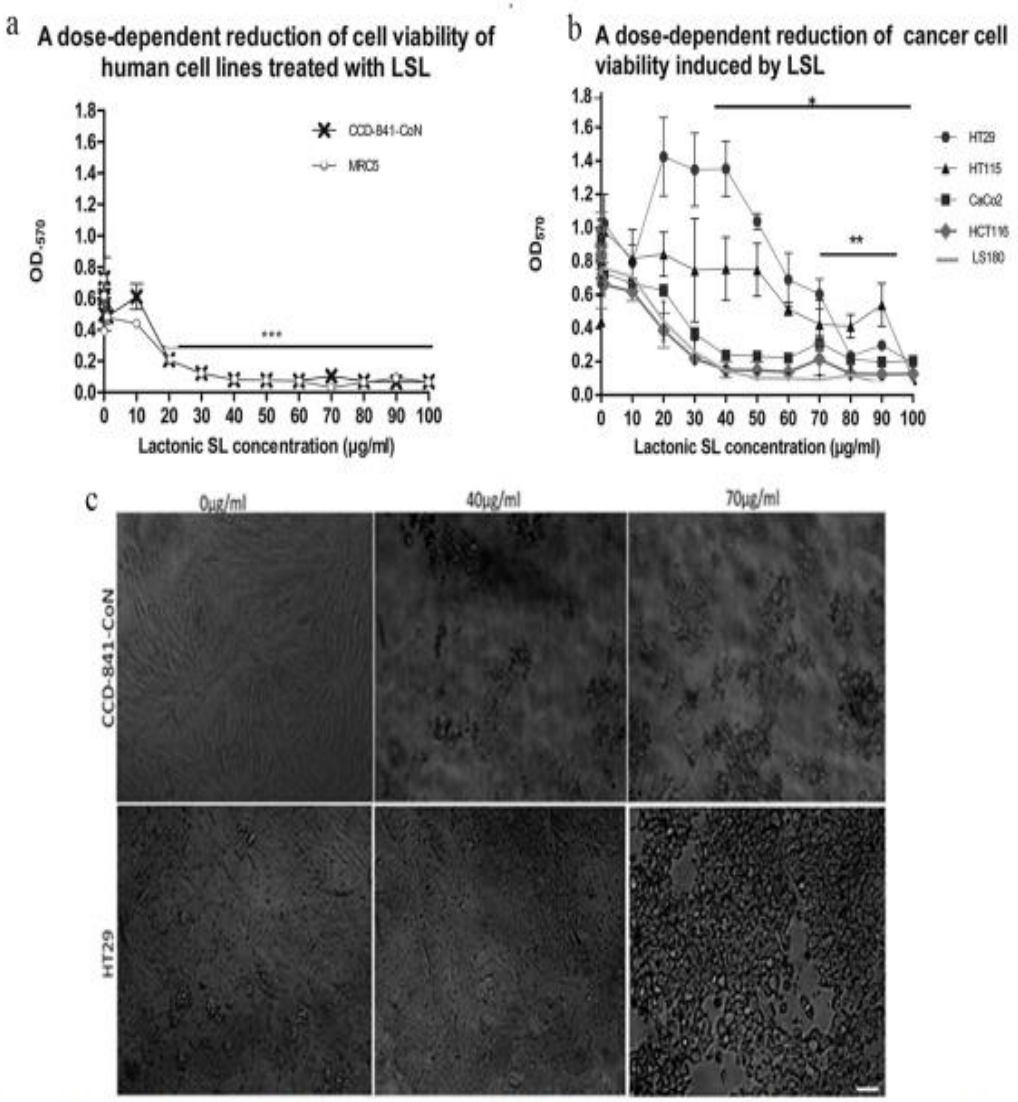


Fig 3. LSL administration causes a dose-dependent effect on cell viability and morphology. (a) Following 24h of treatment with LSL we observed a significant reduction in O.D.570 values at doses between 20–100µg/ml ($p<0.0001$) in the control cell lines CCD-841-CoN and MRC5. (b) There was a significant reduction in O.D.570 values at doses between 30–100µg/ml in HCT116, Caco2 and LS180 ($p < 0.05$) and at doses above 60µg/ml inn HT29 and HT115 cells ($p < 0.001$). Graphs show a representative data set from three independent experimental replicates. Values indicate mean ± SEM ($n = 6$). Statistical significance was assessed one-way ANOVA (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$). (c) Light micrographs from CCD-841-CoN colonic epithelial cells (top) treated with vehicle-only control (left) where cells show a typical bipolar morphology. After treatment with 40µg/ml (central) or 70µg/ml (right) LSL the remaining adherent cells are rounded and there are large regions devoid of cells. In HT29 cultures treated with vehicle-only (bottom right) the cells are densely packed with a cobblestone-like morphology. Following treatment with 40µg/ml LSL (middle), there are no discernable changes in cell morphology, however treatment with 70µg/ml LSL (right) leads to cell rounding and conspicuous cell free areas. Scale bar = 50µm for all images.

doi:10.1371/journal.pone.0156845.g003

alive and 96% of detached cells were dead (S1B–S1E Fig $p < 0.01$). At 100µg/ml, all detached colorectal cancer cells were dead ($p<0.001$).

LSL induce cell death *in vitro*

LSL treatment resulted in a higher proportion of cells undergoing necrosis compared to apoptosis in both normal colonic as well as the four colorectal cancer cell lines we examined

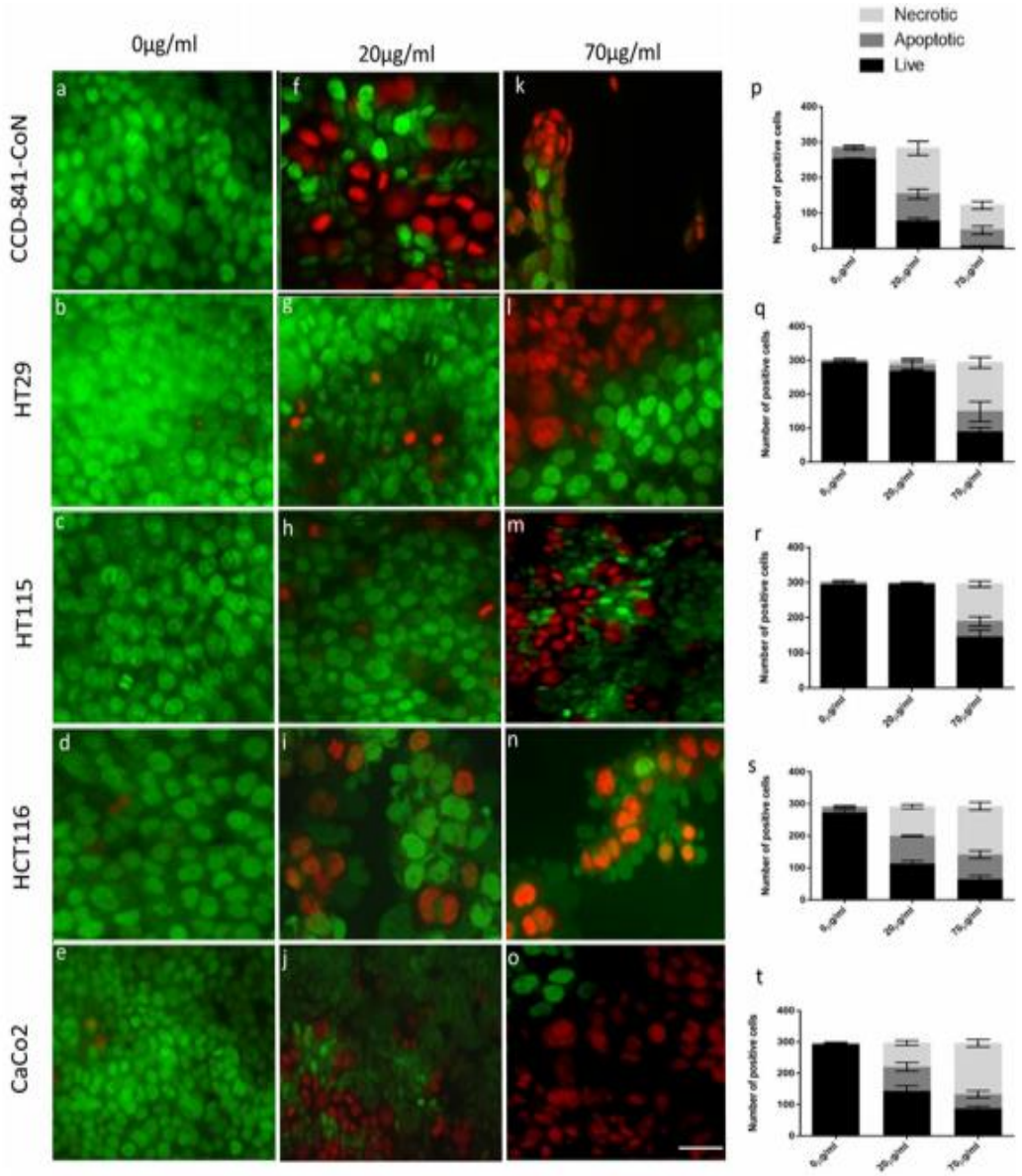


Fig 4. LSL induced both necrosis and apoptosis *in vitro*. Photomicrographs of acridine orange and ethidium bromide stained cultures following treatments with 0 (a-e), 20 (f-j) or 70µg/ml (k-o) LSL and quantification of live, apoptotic or necrotic cells (p-t). The vast number of CCD-841-CoN (a) and cancer cells treated with vehicle control are morphologically viable with a small number showing condensed nuclei (apoptotic). 20µg/ml LSL resulted in necrosis (red/orange clusters) in all cell lines, although CCD-841-CoN (k.p ****p<0.0001) and Caco2 cells (j.o ** p<0.001) were more susceptible. At 70µg/ml, very few adherent CCD-841-CoN cells were observed (k), remaining adhered cells were either necrotic or apoptotic (p). HT29 (l), HT115 (m), HCT116 (n) and Caco2 (o) cells exposed to 70µg/ml LSL all had 50% of cells with morphological features of cells death (q-t) and statistically significant increases in either the numbers of necrotic (*** p<0.0001) or apoptotic cells (**p<0.001) as compared with vehicle only controls.

doi:10.1371/journal.pone.0156845.g004

(Fig 4). In CCD-841-CoN cultures a dose of 20µg/ml LSL resulted in ~70% cell death (Fig 4f), the majority of which were necrotic (Fig 4p). Both HCT116 (Fig 4i) and Caco2 (Fig 4j) were susceptible to cell death at a dose of 20µg/ml LSL, while HT29 (Fig 4g) and HT115 (Fig 4h) were relatively resistant. In CCD-841-CoN cells exposed to 70µg/ml LSL, the few attached cells

available for quantification (Fig 4k) were either necrotic or apoptotic (Fig 4p), whereas in HT29 (Fig 4l), HT115 (Fig 4m), HCT116 (Fig 4n) and Caco2 (Fig 4o) all showed a significant increase in both necrotic ($p < 0.0001$) as well as apoptotic ($p < 0.001$) cells (Fig 4q–4t) when compared with vehicle only (Fig 4a–4e).

Oral administration of LSL to Apc$^{min+/-}$ mice exacerbates tumor development

Genotyping of mice was undertaken following genomic DNA extraction, PCR and subsequent restriction enzyme digestion and electrophoresis; this methodology yielded a single 111bp band for the *wt* allele or dual 111/123 bp alleles (Fig 5a) that are consistent with a heterozygous Apc$^{min+/-}$ mouse. On the basis of genotyping, mice were randomly assigned to either LSL or vehicle-only dosing groups, irrespective of gender. The weights of both *wt* and Apc$^{min+/-}$ mice fed with either vehicle-only control or LSL solutions were not significantly different (25.2g vs 24.9g NS $p < 0.1$) and there were no differences in water (98.2ml vs 99ml; NS, $p > 0.05$) or food (180.7g vs 178g; NS $p > 0.05$) consumption over the duration of the experiment. Dosing of mice for 70 days with LSL also had no effect on the weights of the heart, liver, kidneys or lungs in *wt* mice (data not shown). The gross morphological appearance of unfixed flat mounted ilea from *wt* mice (Fig 5b top) treated with vehicle-only (left) or 50 mg/kg LSL (right) was characterized by a flattened, uniformly smooth mucous epithelium. In vehicle-only treated Apc$^{min+/-}$ mice (Fig 5b; bottom left), there was clear evidence of occult bleeding throughout the ileal segment and numerous polyps (modal diameter 2mm; Fig 5d) compared to *wt* mice. Following treatment with 50mg/kg LSL for 70 days, there is clear evidence of recent bleeding as well as a greater number (vehicle-only = 55.5 ± 3.3 *vs* 50mg/kg LSL = 70.5 ± 7.8; Fig 5c; $p < 0.05$) of larger diameter (modal size 4mm; Fig 5d; $p < 0.001$) polyps throughout the ilea compared to the vehicle only treated Apc$^{min+/-}$ mice ($p < 0.001$). Histological features of sections of *wt* mouse ilea treated with vehicle or 50mg/kg LSL are characterized by evenly spaced, narrow villi with mucoid glands at their base (Fig 5e; top). Sections through Apc$^{min+/-}$ polyps (Fig 5b; bottom) treated with vehicle- only (left) or 50mg/kg LSL (right) reveals a disturbed villous architecture lacking epithelial differentiation.

LSL treatment specifically increases splenic weight and red pulp proportion in the Apc$^{min+/-}$ mouse

The weights and gross morphological appearances of heart, lungs, kidneys and liver were not significantly different between either *wt* and Apc$^{min+/-}$ mice or between mice fed either vehicle only or 50mg/kg LSL (NS, $p > 0.05$; data not shown). Feeding *wt* mice with either vehicle-only or 50mg/kg LSL, also did not affect the wet weights of excised spleen (Fig 6a and 6b; NS, $p > 0.05$). However, spleens from Apc$^{min+/-}$ mice were both larger (Fig 6a) and heavier (Fig 6b; $p < 0.0001$) than those from *wt* mice. Administration of 50mg/kg LSL for 70 days to Apc$^{min+/-}$ mice also resulted in an increase in splenic size (Fig 6a) and weight (Fig 6b; $p < 0.05$). Examination of histological sections from *wt* mouse spleen (Fig 6c; top) revealed conspicuous intensely basophilic areas of white pulp, separated by less dense regions of red pulp in the areas responsible for removal of old or damaged erythrocytes. In Apc$^{min+/-}$ mice the proportion of red pulp was increased compared to *wt* (*c.f.* Fig 6c top and middle; $p < 0.05$). Following treatment with 50mg/kg LSL there was a further increase in red pulp size as compared with vehicle-only controls (*c.f.* Fig 6c middle and bottom $p < 0.05$). Hematocrit values were significantly higher in *wt* than Apc$^{min+/-}$ mice (49.5 ± 0.9 vs 38.1± 1.2; $p < 0.001$). Additionally, feeding Apc$^{min+/-}$ mice with 50 mg/kg LSL for 70 days caused a significant decrease in hematocrit compared to the vehicle-only control (38.1± 1.2 vs 28.2 ± 1.8; $p<0.05$).

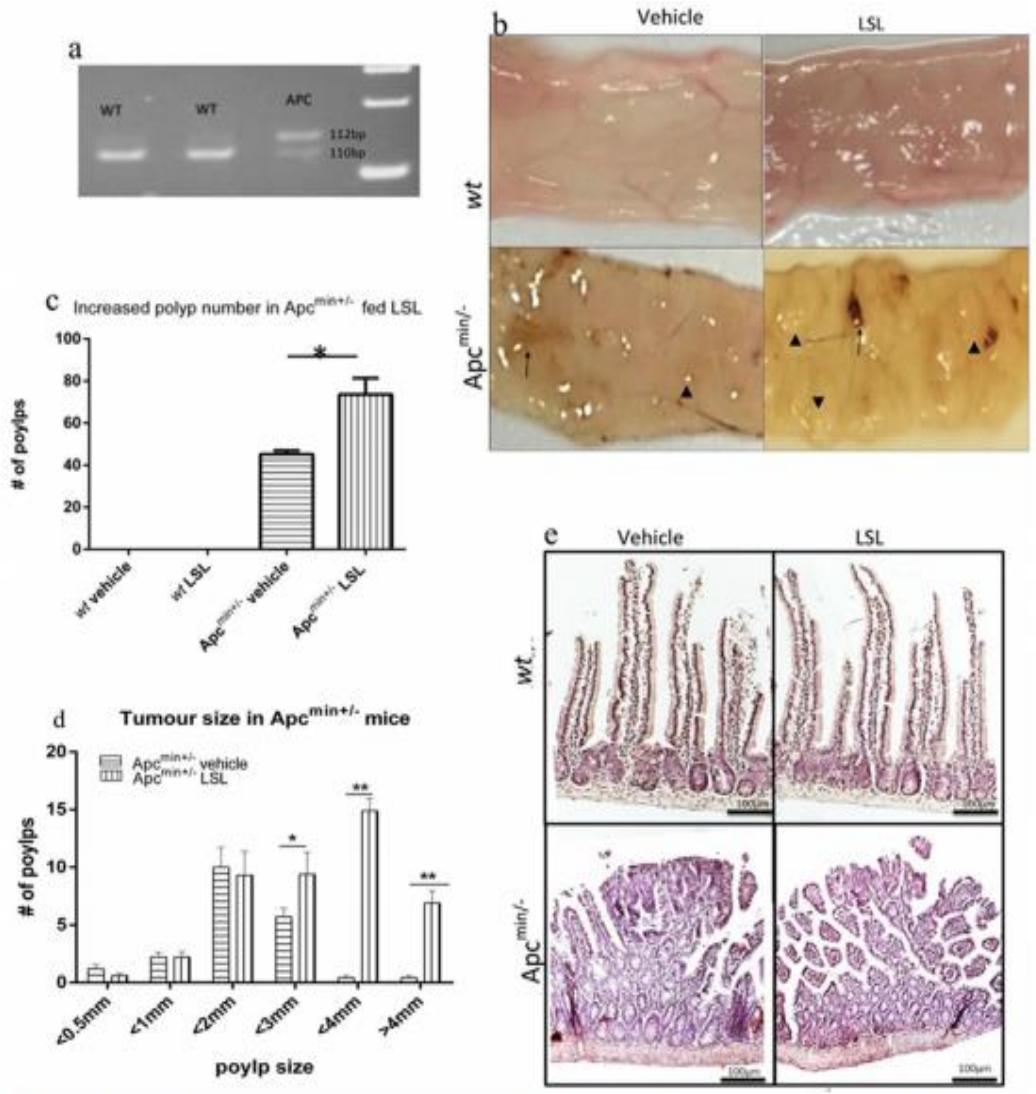


Fig 5. LSL treatment increases tumour number and size in Apc$^{min+/-}$ mice. PCR and restriction enzyme analysis of genomic DNA from wt or Apc$^{min+/-}$ littermates (a); wt band: 111bp, heterozygous Apc$^{min+/-}$ mouse—111 & 123bp. Mice were fed either LSL or vehicle-only every other day for 70 days. Examination of low power images of intestinal segments from wt mice treated with vehicle or 50mg/kg LSL revealed no discernible differences in gross morphology (top). However, in Apc$^{min+/-}$ (bottom), those treated with vehicle-only (left) showed evidence of occult bleeding (arrow) as well as numerous polyps (c arrow heads) with a modal diameter of 2mm (d). In Apc$^{min+/-}$ mice treated with LSL, there was evidence of recent hemorrhage (arrows) as well as significant increases in both polyp number (c;*p <0.01) and size (d;**p < 0.001) compared to vehicle-only controls. Values represent mean ± SEM (n = 10/group). Statistical significance was determined by one-way ANOVA (c) or a student T-tests (d). (e) Histological sections from wt (top) or Apc$^{min+/-}$ (bottom) stained with Haematoxylin and Eosin. Distinct villi in wt treated with vehicle (left) or 50mg/kg LSL are seen. In Apc$^{min+/-}$ littermate mice villous structure is grossly perturbed within polyps in both vehicle-treated mice (bottom left) as well as those treated with 50mg/kg (bottom right).

doi:10.1371/journal.pone.0156845.g005

Discussion

In order to decrease dependency on petrochemical derived surfactants, biosurfactants are increasingly finding use in a variety of applications ranging from industrial and household cleaning reagents through to skin-care products and foodstuffs [8]. The organism with the highest productivity yield of biosurfactants is the pathogenic species *Pseudomonas aeruginosa*, which has made their large scale industrial and health-care use problematic [34]. Modified

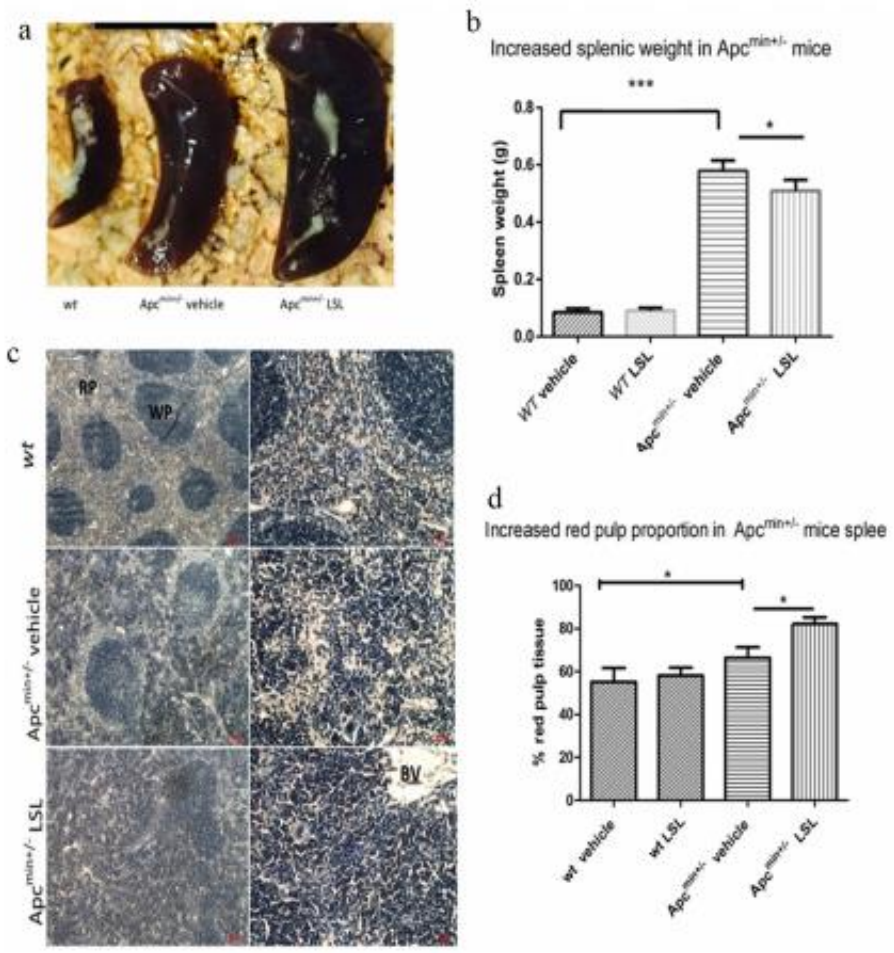


Fig 6. LSL treatment increases spleen size and red pulp area in Apc$^{min+/-}$ mice. a; Photographs of dissected spleen from a wt mouse and Apc$^{min+/-}$ fed with vehicle or LSL. b; Apc$^{min+/-}$ mice had a significantly greater spleen weight compared to wt mice (**** $p<0.00001$). LSL administration resulted in a significant increase in splenic weights compared to the vehicle control mice (* $p<0.01$). Splenic histology from wt mice (6c top), Apc$^{min+/-}$ mice fed vehicle control only (6c middle) and Apc$^{min+/-\ fed}$ LSL (6c bottom). WP = White pulp, RP = Red pulp, BV = Blood vessel. Apc$^{min+/-}$ mice demonstrated altered splenic pathology with increased red pulp regions (hematopoietic rich tissue). LSL fed mice showed a further change in histopathology (*c.f* 6c middle vs bottom) with spleens demonstrating a significant increase in red pulp (* $p<0.05$). Graphs represented of mean ± SEM. Animals per group n = 10. Statistical significance was determined by students T-test.

doi:10.1371/journal.pone.0156845.g006

strains of the yeast *Starmerella bombicola* is a potential commercially viable alternative, as it is non-pathogenic and a high yielding producer of homogenous SL.

In addition to their current commercial uses, SL preparations have previously been reported to have anti-cancer activity based on their ability to reduce the viability of pancreatic [19], lung [21], liver [24] and esophageal cancer cells *in-vitro*. However, these aforementioned studies are difficult to compare, as inter-study variation is significant and the purity as well as homogeneity (proportion of sophorolipid species) is often unreported. Since both purity of SL as well as homogeneity [22] can affect the outcome of biological responses to these molecules, we produced a pure (99% SL) and homogeneous LSL preparation (96% C18:1) for subsequent use in *in vitro* and *in vivo* experiments. SL is formed as a complex mixture with

related species differing by the degree of sophorose acetylation as well as fatty acid length and saturation. This species diversity, coupled with various congeners found in crude SL preparations makes separation and purification difficult, demanding and expensive; however it is vital when considering potential pharma-therapeutic uses. In the past number of years, purification of SL has been achieved by the use of thin layer chromatography, HPLC and column chromatography [35]. The majority of studies investigating the anti-cancer potential of SL separate and purify samples with the use of HPLC, MS or NMR [19, 21] producing data showing the exact composition of the SL to be tested, but the information on purity and composition is often omitted. One notable study [16] which used pure and well characterized SL (92% 18:1 LSL) examined their effects on breast carcinoma cells and found a dose-dependent cytotoxic effect. Here we report on a 96% 18:1 LSL preparation that was used throughout our *in vitro* and *in vivo* experiments. We addressed whether a pure preparation of LSL from *Starmerella bombicola* has a differential and/or dose-dependent effect on transformed adherent cells in comparison to "normal" adherent cells; a highly desirable property for potential cancer chemotherapeutics [36]. We assessed five well characterized colorectal cancer cell lines (HT29, HT115, HCT115, LS180 and CaCo2) in addition to adherent, non-transformed colonic epithelium (CCD-841-CoN) and lung fibroblasts (MRC5). LSL had the capability to discriminate in their ability to induce cell death in these cell types; however, they have a more potent effect against "normal" cells at lower doses (10μg/ml). Only a small number of studies have been carried out looking at the anti-cancer activities of SL isolated from *Starmerella bombicola*, such as the breast cancer line MDA-MB-231 (92% C18:1 LSL). A majority of studies have been carried out using SL produced by *Wickerhamiella domercqiae* which was recently identified as *C. bombicola* after genome sequencing [37]. These cytotoxicity studies [24], also demonstrate similar potent effect of SL from doses ranging from 40μg/ml– 2mg/ml. The wide range of dose efficacy may be partially explained by the differences in SL species and uncharacterized mixtures. The repeatability and high level of consistency in the data from our in vitro and in vivo studies is consistent with our conclusions on the biological activity of our LSL sample, although we cannot exclude the possibility that the 3.8% ASL found within our SL mixture has a co-incident biological activity.

LSL mixtures had no effect on circulating (non-adherent) blood monocytes, although their comparison with adherent pancreatic tumor cells is spurious. Other non-transformed cell lines examined in the literature include the uncharacterized, and not readily available HL7702 and the 'Chang' liver cells [24, 25] believed to derived from normal liver, but later found to be HeLa contaminated [38].

We determined that colorectal cells supplemented with 40–70μg/ml LSL begin to die after 24hr *in vitro*. The predominant type of cell death observed, following ethidium bromide/acridine orange staining, was necrosis. This occurred at doses of 70μg/ml in the cancer cell lines and 20μg/ml in the normal cell lines. Necrosis is a type of unregulated programmed cell death [39], characterized by the disruption of the lipid membrane resulting in the leakage of intracellular proteins, reduction in ATP and cell lysis thus provoking an immune response [40]. SL induced necrosis has been demonstrated in other cell lines, as quantified by LDH release: such as HPAC [16] and the HL-60 leukemic cell line [41].

The induction of necrosis in various cell lines (including those described in this study) likely occurs *via* the intercalation of biosurfactants into the lipid bilayer as has been previously documented [42]. Koley *et al.*, 2010 explained that, at a cell-line specific minimal concentration, surfactants integrate into the cell lipid membrane, resulting in carbon chain structural rearrangement. High doses induce tension at the interfacial region of the bilayer, resulting in phospholipid dehydration which affects lipid stability, cellular adhesion and function [43]. This

ultimately results in cell death [44], which is evident in studies of SL induced membrane disruption in sperm [18].

Studies investigating the therapeutic potential of SL *in vivo* are limited with the exception of sepsis models. SL mixtures reduce mortality in rats with experimentally induced sepsis via cecum puncture. However in comparison to the natural mixtures—LSL has caused an unexpected increase in the mortality rate in the septic rats at the same dose [20].

The Apc$^{min+/-}$ mouse is a popular animal model to investigate the correlation between food, genetics and chemotherapeutic in the development of intestinal adenomatous neoplasms (polyps). These mice have a life span of <150 days due to secondary consequences of the disease (extensive bleeding of colonic polyps accompanied by anemia) thus making it an ideal and quick model to study the effects of compounds [28, 29, 45, 46]. Oral administration was chosen as the ideal route of administration, in contrast to a traumatic abdominal injury, as it allows the LSL to have direct access to the gut epithelium and polyps to exert their biological effect. Considering the ability of SL mixtures to reduce cancer cell viability, it is surprising that we could find no reports of the *in vivo* use of these SL in established pre-clinical models of cancer development. As our studies indicated an effect of LSL on a range of colorectal cancer cell lines at dosages that would be tolerated for oral administration, we hypothesized that long-term administration would slow progression of colorectal tumors in the Apc$^{min+/-}$ mouse model.

The results show that orally administered purified LSL did not decrease polyp development, but instead caused the exacerbated growth of adenomatous polyps in the intestinal pre-cancerous Apc$^{min+/-}$ mouse model. LSL treatment also increased the size (volume) of the polyps which is currently used as an indicator of tumor burden [47].

The use of other markers is useful in determining disease progression. Apcmi$^{min+/-}$ naturally present with an enlarged spleen and reduced hematocrit as a result of colorectal bleeding [45, 46]. Our study showed that LSL administration resulted in a further increase in spleen size and reduced hematocrit compared to the vehicle control mice. The increase in spleen size may be due to the role it has in clearing out dead and defective erythrocytes [48]. The effect has been documented with other drug administration in mice such as benzo(a)pyrene, an immunomodulatory drug [49].

In conclusion, LSL do not discriminate in their ability to induce cell death between transformed and normal cell lines, as well as increasing progression in the pre-clinical Apc$^{min+/-}$ mouse model. This study is therefore instructive in urging caution concerning the interpretation of *in vitro* studies examining potential anti-tumor effects of purified preparations of LSL and SL in general.

Supporting Information

S1 Fig. LSL treatment results in the detachment of dead cells. Quantification of live or dead cells using propidium iodide and Syto 9 staining on cells extracted from the supernatant of cultures treated with 0, 50 or 100μg/ml of LSL. Following treatment with 50μg/ml LSL, CCD-84l CoN (a), all detached cells in the supernatant were dead (*** $p < 0.001$). At the same concentration, a small number of colorectal cancer cells (b-e) were alive (4%) while the remainder were dead (96% *$p < 0.01$). In CCD-84l-CoN control cultures and colorectal cancer cells exposed to 100μg/ml LSL, 100% of cells counted in the supernatant were dead (*** $p < 0.0001$). Graphs representative of mean ± SEM. Significance was calculated using a student's t-test and one-way ANOVA.
(TIF)

Author Contributions

Conceived and designed the experiments: BC CAM. Performed the experiments: BC HL SLKWR INAVB. Analyzed the data: BC HL SLKWR INAVB RM IMB CAM. Wrote the paper: BC HL SLKWR INAVB RM IMB CAM.

References

1. Mulligan CN. Environmental applications for biosurfactants. Environmental pollution. 2005; 133 (2):183–98. PMID: 15519450
2. Rodrigues LR, Teixeira JA. Biomedical and therapeutic applications of biosurfactants. Biosurfactants: Springer; 2010. p. 75–87.
3. Fracchia L, Cavallo M, Martinotti MG, Banat IM. Biosurfactants and bioemulsifiers biomedical and related applications—present status and future potentials. Biomedical science, engineering and technology. 2012; 14:326–35.
4. Bognolo G. Biosurfactants as emulsifying agents for hydrocarbons. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 1999; 152(1):41–52.
5. Finnerty WR. Biosurfactants in environmental biotechnology. Current Opinion in Biotechnology. 1994; 5(3):291–5.
6. Montoneri E, Boffa V, Savarino P, Tambone F, Adani F, Micheletti L, et al. Use of biosurfactants from urban wastes compost in textile dyeing and soil remediation. Waste management. 2009; 29(1):383–9. doi: 10.1016/j.wasman.2008.01.011 PMID: 18346886
7. Kralova I, Sjöblom J. Surfactants used in food industry: a review. Journal of Dispersion Science and Technology. 2009; 30(9):1363–83.
8. Nitschke M, Costa S. Biosurfactants in food industry. Trends in Food Science & Technology. 2007; 18 (5):252–9.
9. Levin J, Miller R. A guide to the ingredients and potential benefits of over-the-counter cleansers and moisturizers for rosacea patients. The Journal of clinical and aesthetic dermatology. 2011; 4(8):31. PMID: 21909456
10. Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, et al. Microbial biosurfactants production, applications and future potential. Applied microbiology and biotechnology. 2010 Jun; 87 (2):427–44. PMID: 20424836. doi: 10.1007/s00253-010-2589-0
11. Van Bogaert IN, Saerens K, De Muynck C, Develter D, Soetaert W, Vandamme EJ. Microbial production and application of sophorolipids. Applied microbiology and biotechnology. 2007; 76(1):23–34. PMID: 17476500
12. Chen J, Song X, Zhang H, Qu Y. Production, structure elucidation and anticancer properties of sophorolipid from *Wickerhamiella domercqiae*. Enzyme and microbial technology. 2006; 39(3):501–6.
13. Cavalero DA, Cooper DG. The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. Journal of biotechnology. 2003; 103 (1):31–41. PMID: 12770502
14. Bluth MH, Kandil E, Mueller CM, Shah V, Lin Y-Y, Zhang H, et al. Sophorolipids block lethal effects of septic shock in rats in a cecal ligation and puncture model of experimental sepsis*. Critical care medicine. 2006; 34(1):E188.
15. Napolitano LM. Sophorolipids in sepsis: Antiinflammatory or antibacterial?*. Critical care medicine. 2006; 34(1):258–9. PMID: 16374196
16. Ribeiro IA, Faustino C, Guerreiro PS, Frade RF, Bronze MR, Castro MF, et al. Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer cells. Journal of Molecular Recognition. 2015; 28(3):155–65. doi: 10.1002/jmr.2403 PMID: 25647712
17. Shao L, Song X, Ma X, Li H, Qu Y. Bioactivities of sophorolipid with different structures against human esophageal cancer cells. Journal of Surgical Research. 2012; 173(2):286–91. doi: 10.1016/j.jss.2010.09.013 PMID: 21035135
18. Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, et al. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrobial agents and chemotherapy. 2005; 49(10):4093–100. PMID: 16189085
19. Fu SL, Wallner SR, Bowne WB, Hagler MD, Zenilman ME, Gross R, et al. Sophorolipids and their derivatives are lethal against human pancreatic cancer cells. Journal of Surgical Research. 2008; 148 (1):77–82. doi: 10.1016/j.jss.2008.03.005 PMID: 18570934

20. Hardin R, Pierre J, Schulze R, Mueller CM, Fu SL, Wallner SR, et al. Sophorolipids improve sepsis survival: effects of dosing and derivatives. Journal of Surgical Research. 2007; 142(2):314–9. PMID: 17719064

21. Chen J, Song X, Zhang H, Qu Y. Production, structure elucidation and anticancer properties of sophorolipid from Wickerhamiella domercqiae. Enzyme and microbial technology. 2006; 39(3):501–6.

22. Cavalero DA, Cooper DG. The effect of medium composition on the structure and physical state of sophorolipids produced by Candida bombicola ATCC 22214. Journal of biotechnology. 2003; 103 (1):31–41. PMID: 12770502

23. Manzke C. Prozeßabha¨ngige aufarbeitung und HPLCgestu¨tze analyse von sophoroselipiden aus Candida bombicola. TU Braunschweig, Germany 1999.

24. Chen J, Song X, Zhang H, Qu Y-b, Miao J-y. Sophorolipid produced from the new yeast strain Wickerhamiella domercqiae induces apoptosis in H7402 human liver cancer cells. Applied microbiology and biotechnology. 2006; 72(1):52–9. PMID: 16528516

25. Ma X, Li H, Song X. Surface and biological activity of sophorolipid molecules produced by Wickerhamiella domercqiae var. sophorolipid CGMCC 1576. Journal of colloid and interface science. 2012; 376 (1):165–72. doi: 10.1016/j.jcis.2012.03.007 PMID: 22459028

26. Hagler M, Smith-Norowitz T, Chice S, Wallner S, Viterbo D, Mueller C, et al. Sophorolipids decrease IgE production in U266 cells by downregulation of BSAP (Pax5), TLR-2, STAT3 and IL-6. Journal of Allergy and Clinical Immunology. 2007; 119(1):S263.

27. Bluth M, Fu S, Fu A, Stanek A, Smith-Norowitz T, Wallner S, et al. Sophorolipids decrease asthma severity and ova-specific IgE production in a mouse asthma model. Journal of Allergy and Clinical Immunology. 2008; 121(2):S2.

28. Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD, Gould MN. ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. Proceedings of the National Academy of Sciences. 1993; 90(19):8977–81.

29. Su L-K, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science. 1992; 256(5057):668–70. PMID: 1350108

30. Roelants SL, Ciesielska K, De Maeseneire SL, Moens H, Everaert B, Verweire S, et al. Towards the industrialization of new biosurfactants: Biotechnological opportunities for the lactone esterase gene from Starmerella bombicola. Biotechnology and bioengineering. 2015.

31. Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. BMC biotechnology. 2005; 5(1):12.

32. Workman P, Aboagye E, Balkwill F, Balmain A, Bruder G, Chaplin D, et al. Guidelines for the welfare and use of animals in cancer research. British journal of cancer. 2010; 102(11):1555–77. doi: 10.1038/sj.bjc.6605642 PMID: 20502460

33. Casteleyn C, Rekecki A, Van der Aa A, Simoens P, Van den Broeck W. Surface area assessment of the murine intestinal tract as a prerequisite for oral dose translation from mouse to man. Laboratory animals. 2010; 44(3):176–83. doi: 10.1258/la.2009.009112 PMID: 20007641

34. Lang S, Wullbrandt D. Rhamnose lipids—biosynthesis, microbial production and application potential. Applied Microbiology and Biotechnology. 1999; 51(1):22–32. PMID: 10077819

35. Nunez A, Ashby R, Foglia T, Solaiman D. Analysis and characterization of sophorolipids by liquid chromatography with atmospheric pressure chemical ionization. Chromatographia. 2001; 53(11–12):673–7.

36. de Oliveira MR, Magri A, Baldo C, Camilios-Neto D, Minucelli T, Celligoi MAPC. Review: Sophorolipids A Promising Biosurfactant and it's Applications.

37. Li J, Li H, Li W, Xia C, Song X. Identification and characterization of a flavin-containing monooxygenase MoA and its function in a specific sophorolipid molecule metabolism in Starmerella bombicola. Applied microbiology and biotechnology. 2016; 100(3):1307–18. doi: 10.1007/s00253-015-7091-2 PMID: 26512005

38. Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. Nature Reviews Cancer. 2002; 2 (4):315–9. PMID: 12001993

39. Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, et al. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell proliferation. 2012; 45(6):487–98. doi: 10.1111/j.1365-2184.2012.00845.x PMID: 23030059

40. Robbins SL, Kumar V, Abbas AK, Aster JC. Robbins basic pathology: Elsevier Health Sciences; 2012.

41. Isoda H, Kitamoto D, Shinmoto H, Matsumura M, Nakahara T. Microbial extracellular glycolipid induction of differentiation and inhibition of the protein kinase C activity of human promyelocytic leukemia cell line HL60. Bioscience, biotechnology, and biochemistry. 1997; 61(4):609–14. PMID: 9145519

42. Fracchia L, Banat JJ, Cavallo M, Ceresa C, Banat IM. Potential therapeutic applications of microbial surface-active compounds. AIMS Bioengineering, 2015 Volume 2, Issue 3, 144–162: doi: 10.3934/bioeng.2015.3.144

43. Fracchia L, Banat IM, Martinotti MG, Cavallo M. Biosurfactants and bioemulsifiers biomedical and related applications-present status and future potentials: INTECH Open Access Publisher; 2012. Chapter 14, pg 325–370.

44. Koley D, Bard AJ. Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). Proceedings of the National Academy of Sciences. 2010; 107(39):16783–7.

45. Yekkala K, Baudino TA. Inhibition of intestinal polyposis with reduced angiogenesis in ApcMin/+ mice due to decreases in c-Myc expression. Molecular Cancer Research. 2007; 5(12):1296–303. doi: 10.1158/1541-7786.MCR-07-0232 PMID: 18171987

46. You S, Ohmori M, Peña MMO, Nassri B, Quiton J, Al-Assad ZA, et al. Developmental abnormalities in multiple proliferative tissues of ApcMin/+ mice. International journal of experimental pathology. 2006; 87(3):227–36. PMID: 16709231

47. Goodlad R, Ryan A, Wedge S, Pyrah I, Alferez D, Poulsom R, et al. Inhibiting vascular endothelial growth factor receptor-2 signaling reduces tumor burden in the ApcMin/+ mouse model of early intestinal cancer. Carcinogenesis. 2006; 27(10):2133–9. PMID: 16782971

48. Crosby WH. Normal functions of the spleen relative to red blood cells: a review. Blood. 1959; 14 (4):399–408. PMID: 13638340

49. Petroianu A. Drug-induced splenic enlargement. Expert Opinion on Drug Safety: 2007 march- 2007. Report No. 10.1517/14740338.6.2.199.