Lactonic Sophorolipids Increase Tumor Burden in Apc\textsuperscript{min+/-} Mice

Breedge Callaghan\textsuperscript{1}, Helen Lydon\textsuperscript{1}, Sophie L. K. W. Roelants\textsuperscript{2}, Inge N. A. Van Bogaert\textsuperscript{2}, Roger Marchant\textsuperscript{1}, Ibrahim M. Banat\textsuperscript{1}, Christopher A. Mitchell\textsuperscript{1,*}

\textsuperscript{1} Biomedical Sciences Research Institute, Centre for Molecular Biosciences, Ulster University, Coleraine, BT52 1SA, United Kingdom, \textsuperscript{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\textsuperscript{‘} and C6\textsuperscript{”} 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\textit{ 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 tumor cell lines \textit{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 \textit{in vitro} and \textit{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 \textit{in vitro}. Oral administration of vehicle-only; or lactonic sophorolipids (50 mg/kg for 70 days), to Apc\textsuperscript{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.

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 $\beta$-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 $\beta$-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.

![Lactonic Sophorolipid](https://doi.org/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<sup>min/-</sup> 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<sup>4</sup> 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 10μg/ml acridine orange (Sigma-Aldrich Company Ltd, Dorset, UK) and 1μg/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 fluorescence 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<sup>min-/+</sup> 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<sup>min-/+</sup>/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<sup>min-</sup> specific primers (100μm each) (Forward: TCT CGT TCT GAG AAA GAC AGA AGC T, Reverse: TGA TAC TTC TCC CAA AGC TTC TTT GCC 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 HindIII 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,
The presence of a single band at 111bp indicated a wt mouse, while an additional band at 123bp indicated a heterozygous Apc\textsuperscript{min+/-} mouse.

**Sophorolipid dosing**

At five weeks of age, equal numbers of wt and Apc\textsuperscript{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 (pH 7.4). 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, 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 \textit{in vitro} data was determined using either one-way ANOVA or student’s \textit{t}-test using GraphPad Prism (GraphPad software, San Diego, USA). All comparisons between \textit{in vivo} groups were assessed using a student’s \textit{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 \textit{in vitro} and \textit{in vivo} studies (Fig 1), we employed the \textit{S. bombicola} oe \textit{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.
(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 and 70 μg/ml there were large areas devoid of cells, with remaining adherent cells displaying a shrunked 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
alive and 96% of detached cells were dead (S1B–S1E Fig p < 0.01). At 100 μg/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.
In CCD-841-CoN cultures, a dose of 20 μg/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 20 μg/ml LSL, while HT29 (Fig 4g) and HT115 (Fig 4h) were relatively resistant. In CCD-841-CoN cells exposed to 70 μg/ml LSL, the few adherent cells remaining adhered were either necrotic or apoptotic (Fig 4k). HT29 (Fig 4l), HT115 (Fig 4m), HCT116 (Fig 4n) and Caco2 (Fig 4o) cells exposed to 70 μg/ml LSL all had 50% of cells with morphological features of cell 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.

(Fig 4). 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). 20 μg/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 70 μg/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 70 μg/ml LSL all had 50% of cells with morphological features of cell 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. 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 70 μg/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). 20 μg/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 70 μg/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 70 μg/ml LSL all had 50% of cells with morphological features of cell 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.
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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{min/+} mouse**

The weights and gross morphological appearances of heart, lungs, kidneys and liver were not significantly different between either wt and Apc\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{min/+} mice (49.5 ± 0.9 vs 38.1± 1.2; p <0.001). Additionally, feeding Apc\textsuperscript{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).
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].

**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
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<sub>min+/-</sub> 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<sub>min+/-</sub> 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<sub>min+/-</sub> 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. Apc<sub>mi+/-</sub> 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<sub>min+/-</sub> 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. Boghno G. Biosurfactants as emulsifying agents for hydrocarbons. Colloids and Surfaces A: Physico-chemical 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). 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 MF, 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: 21059135
18. Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagyer 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, Zenitman 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
Li J, Li H, Li W, Xia C, Song X. Identification and characterization of a flavin-containing monooxygenase.

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. Cavalerò 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. Prozeßabhangige aufarbeitung und HPLCgestützte 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. ApoMin, 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 its Applications.

37. Li J, Li H, Li W, Xia C, Song X. Identification and characterization of a flavin-containing monoxygenase 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, Shimoto 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 D, Alferz R, 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.