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Breedge Callaghan, Matthew Twigg, Niki Baccile, Inge van Bogaert, Roger Marchant, Christopher Mitchell, Ibrahim Banat

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1 **Microbial sophorolipids inhibit colorectal tumour cell growth *in vitro* and restore haematocrit in Apc<sup>min+/-</sup>**  
2 **mice**

3

4 Breedge Callaghan<sup>1</sup>, Matthew S. Twigg<sup>1</sup>, Niki Baccile<sup>2</sup>, Inge N. A. Van Bogaert<sup>3</sup>, Roger Marchant<sup>1</sup>, Christopher  
5 A. Mitchell<sup>1,\*</sup> & Ibrahim M. Banat<sup>1,\*</sup>

6

7 1. School of Biomedical Sciences, Ulster University, Coleraine, BT52 1SA, U.K.

8 2. Sorbonne Universités, UPMC Univ Paris 06, CNRS, Collège de France UMR 7574, Chimie de la Matière  
9 Condensée de Paris, UMR 7574, 75005 Paris, France.

10 3. Centre for Synthetic Biology, Department of Biotechnology, Ghent University, Coupure Links 653, 9000  
11 Ghent, Belgium.

12

13 BC : ORCID - 0000-0002-0798-2540

14 MST : ORCID - 0000-0002-8601-1960

15 NB : ORCID - 0000-0003-3142-3228

16 INAVB : ORCID - 0000-0001-8144-6466

17 RM : ORCID - 0000-0001-5213-229X

18 IMB : ORCID - 0000-0001-8964-2840

19 CM : ORCID - 0000-0002-4422-7499

20

21 \*Corresponding Authors – Ibrahim Banat and Christopher Mitchell

22 Email: im.banat@ulster.ac.uk, ca.mitchell@ulster.ac.uk

23 Tel: +44 28 7012 3062, +44 28 7012 4089

24

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26

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30 826.

31 **Abstract**

32

33 Sophorolipids are glycolipid biosurfactants consisting of a carbohydrate sophorose head with a fatty acid tail and  
34 exist in either an acidic or lactonic form. Sophorolipids are gaining interest as potential cancer chemotherapeutics  
35 due to their inhibitory effects on a range of tumour cell lines. Currently, most anti-cancer studies reporting the  
36 effects of sophorolipids have focused on lactonic preparations with the effects of acidic sophorolipids yet to be  
37 elucidated. We produced a 94% pure acidic sophorolipid preparation which proved to be non-toxic to normal  
38 human colonic and lung cells. In contrast we observed a dose-dependent reduction in viability of colorectal cancer  
39 lines treated with the same preparation. Acidic sophorolipids induced apoptosis and necrosis, reduced migration  
40 and inhibited colony formation in all cancer cell lines tested. Furthermore, oral administration of 50 mg kg<sup>-1</sup> acidic  
41 sophorolipids over 70 days to Apc<sup>min/+</sup> mice was well tolerated and resulted in an increased haematocrit, as well  
42 as reducing splenic size and red pulp area. Oral feeding did not affect tumour numbers or sizes in this model. This  
43 is the first study to show that acidic sophorolipids dose-dependently and specifically reduces colon cancer cell  
44 viability in addition to reducing tumour-associated bleeding in the Apc<sup>min/+</sup> mouse model.

45

46 **Keywords:** Sophorolipids, Acidic, Colorectal, Cancer, Apc<sup>min/+</sup>

47

48 **Statements and Declarations**

49

50 Competing interests: The authors declare that they have no competing interests.

51

52 Ethical approval: The article does not contain any studies with human participants where ethical was required.

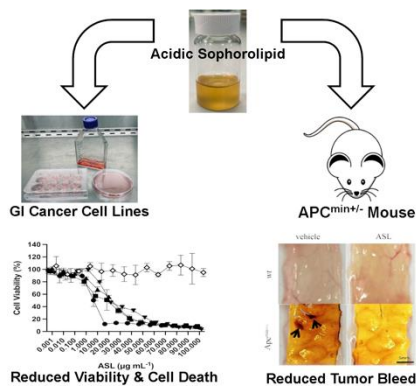
53 Experiments using animals were carried out after obtaining ethical approval and under licence in accordance with  
54 the Animals (Scientific Procedures) Act 1986, U.K. Further detail is provided in the material and methods section.

55

56 Data availability statement: The datasets generated during and/or analysed during the current study are available  
57 from the corresponding authors on reasonable request.

58

59 **Graphical Abstract**



60

61

62 **Key Points**

63

64 Acidic sophorolipids are produced by yeast species such as *Starmerella bombicola*.

65 Acidic sophorolipids selectively killed colorectal cells with no effect on healthy gut epithelia.

66 Acidic sophorolipids reduced tumour associated gut bleed in a colorectal mouse model.

67

68 **Introduction**

69

70 Familial Adenomatous Polyposis (FAP) is an autosomal dominant hereditary form of Colorectal cancer was  
 71 responsible for over 10% of global cancer incidence and 9.4% of global cancer related deaths in 2020 (Ferlay et  
 72 al. 2020). FAP is characterised by the development of numerous adenomas along the colorectal tract. Currently,  
 73 the gold standard for treatment of FAP is surgery, followed by adjuvant chemotherapy (O’Connell et al. 2010).  
 74 Adjuvant chemotherapy does not however discriminate between normal and transformed tissue, which leads to a  
 75 variety of potentially serious complications including cardiotoxicity, immune dilapidation, and neurotoxicity  
 76 (Morgan and Rubin 1998; Mazevet et al. 2013). To allay this problem, compounds that are non-toxic, orally  
 77 tolerated and specifically target epithelial neoplastic cells in the intestinal tract could have great potential in  
 78 delaying progression of intestinal neoplasms; in particular, those that are associated with progression to these  
 79 colorectal cancers (Kelloff et al. 1994; D’Incalci et al. 2005).

80 There is an increasing body of research demonstrating compounds derived from natural sources such as  
 81 plants and microbial secondary metabolites have preventative or targeted anti-cancer activity (Fridlender et al.  
 82 2015; Khalifa et al. 2019). An example that is already in widespread in cancer treatment is Paclitaxel, first derived  
 83 from the bark of the yew tree (*Taxus baccata*) (Stierle et al. 1993) and is now used for the treatment of several  
 84 different cancer types including ovarian, oesophageal, breast, lung and pancreatic cancer (Barbuti and Chen 2015).

85 Sophorolipids are a class of naturally occurring glycolipids produced by yeast species such as *Starmerella*  
86 *bombicola* (Van Bogaert et al. 2007; Roelants et al. 2019). Comprising a hydrophilic sophorose moiety covalently  
87 linked to hydrophobic hydroxylated fatty acid tails ranging between 16 and 18 carbons in length, sophorolipid  
88 congeners are produced in two main forms either lactonic (LSL) or acidic (ASL) (Fig. S1) (Banat et al. 2010;  
89 Marchant and Banat 2012a; Marchant and Banat 2012b). During fermentation yeast species generate mixtures of  
90 these structurally different congeners (Van Bogaert *et al.*, 2007). The anti-cancer properties of sophorolipids have  
91 received a lot of attention in recent years. Sophorolipids and their synthetically synthesised derivatives have shown  
92 *in vitro* cytotoxic effects in human pancreatic (HPAC), liver (H7402), lung (A549), brain (LN229, HNCG-2),  
93 oesophageal (KYSE109, KYSE450), breast, cervical (HeLa), leukaemic (HL60, K562) and melanoma (SK-  
94 MEL-28) cell lines (Chen et al. 2006a; Chen et al. 2006b; Fu et al. 2008; Dhar et al. 2011; Shao et al. 2012;  
95 Ribeiro et al. 2015; Li et al. 2017; Ceresa et al. 2021; Adu et al. 2022). Sophorolipids have also shown potential  
96 tumour shrinking capability in an *in vivo* model of cervical cancer (Li et al. 2017). However, to date, very few *in*  
97 *vivo* bioactivity studies have been reported for sophorolipid mixtures. A small number of toxicology experiments  
98 have shown that sophorolipids are non-irritating when topically applied to the skin and eyes of rabbits and non-  
99 toxic when administered orally to either mice or rats (Ikeda et al. 1986; Callaghan et al. 2016). Sophorolipid  
100 mixtures have also been observed to reduce inflammation and reduce mortality rates in a rat model of severe  
101 abdominal sepsis as well as decreasing IgE levels in a murine asthma model (Hardin et al. 2007; Bluth et al. 2008).

102         Although sophorolipids show promising anti-cancer activity *in vitro*, the gross composition and  
103 percentage of congeners within the sophorolipid mixture used in most investigations are not fully disclosed. It can  
104 be assumed that LSL enriched preparations are more common, due to the preponderance of LSL's in comparison  
105 to ASL's within naturally occurring sophorolipid mixtures (Van Bogaert et al. 2011; Marchant and Banat 2012a).  
106 The composition of sophorolipid preparations is an important factor to consider when investigating the targeted  
107 or preventative anti-cancer effect of these molecules. The reasoning for this is twofold; firstly, to reduce toxicity  
108 resulting from contaminants in crude preparations and, natural products being investigated must be composed  
109 completely of highly purified biologically active agent (Beutler 2019; Adu et al. 2022). In the case of paclitaxel  
110 and other naturally derived agents, this has necessitated the synthetic production in the laboratory of the active  
111 agent with targeted anti-cancer properties (Flam 1994). Secondly, and arguably more important, there is  
112 significant difference in the functional properties attributed to LSL and ASL congeners. LSL's have strong  
113 antimicrobial action that is not seen in ASL's (Van Bogaert et al. 2007; Elshikh et al. 2017). Previously, we have  
114 shown that a highly purified preparation of LSL results in exacerbation of adenomatous tumour growth in the

115 intestinal tract of a colorectal cancer murine model, ( $Apc^{min/+}$  mouse), with secondary consequences including  
116 splenomegaly and reduced haematocrit levels (Callaghan et al. 2016).

117 To date, the anti-cancer bioactivity of purified ASL's have only been investigated within melanoma cell  
118 lines (Adu et al. 2022). In contrast to our previous work with LSL, an ASL specific targeted anti-cancer effect  
119 would be advantageous for several reasons. In comparison to LSL congeners, ASL's have reduced production  
120 costs as they are the first congeners to be produced in the bioreactor when less favourable conditions that require  
121 a reduced energy requirement are present (Casas and García-Ochoa 1999; Sarubbo et al. 2022). The proportion of  
122 ASL within enriched preparations can be improved when the medium is supplemented with polyunsaturated fatty  
123 acids and this can be further increased via simple hydrolysis of the sophorolipid mixtures which increases the  
124 number of open ring structures (Van Bogaert et al. 2011). Alternatively, a modified strain lacking the lactonising  
125 enzyme can be applied. Yet, in contrast to the hydrolysis strategy, acetylation of the glucose moieties is still  
126 possible (Roelants *et al.*, 2016). ASL's have enhanced solubility when compared to other sophorolipid congeners,  
127 which may prove useful for their use as pharmaceuticals, as they can be dissolved in saline instead of potentially  
128 toxic solvents such as DMSO (Baccile et al. 2016). Both the solubility as well as enhanced foaming attributes of  
129 ASL's contribute to their preferred use in the food, cosmetic and the bioremediation industries (Roelants et al.  
130 2016; Naughton et al. 2019; Adu et al. 2020).

131 In this study we investigated the targeted therapeutic effect of purified ASL *in vitro* in human colorectal  
132 cancer cell lines and their ability to inhibit tumour growth in  $Apc^{min/+}$  mice. The  $Apc^{min/+}$  mouse model of FAP  
133 was used as it recapitulates key pathological features of the human disease and provides a useful tool to investigate  
134 the effects of genetics, diet, and therapeutic drugs on tumorigenesis in the gastrointestinal tract (Hinoi et al. 2007).  
135 Like human FAP, it was first noted that  $Apc^{min/+}$  mice develop adult-onset anaemia with haematocrit levels <45%,  
136 the passage of bloody stools, and have a reduced life span (Moser et al. 1995). Additionally, post-mortem analysis  
137 revealed the growth of numerous tumours along the small intestine (Moser et al. 1995). The  $Apc^{min/+}$  mouse model  
138 has also been widely used to test the effects of chemotherapeutics on tumour growth and development such as  
139 NSAIDs (Aspirin) (Reuter et al. 2002). In addition, the  $Apc^{min/+}$  mouse model has also been used to develop a  
140 further understanding of clinically used chemotherapeutic drugs such as 5-fluorouracil, which is widely used in  
141 the treatment of CRC (Tucker et al. 2002). Therefore, we hypothesize that a highly purified and well characterised  
142 preparation of ASL (94% pure diacetylated) will selectively inhibit colorectal tumour growth *in vitro* and delay  
143 disease progression in the  $Apc^{min/+}$  mouse model.

144

145 **Material and Methods**

146

147 ASL production and purification.

148

149 ASL's used in this study were generated in house from a crude precursor sophorolipid mixture, (*Sopholiance S*,  
150 Batch N°11103A), purchased from *Givaudan* (Vernier, Switzerland). ASL congeners were obtained according to  
151 a method described by Baccile *et al.* (2013). In brief, sophorolipids were purified from *Sopholiance S* via liquid  
152 phase extraction in acidified ethyl acetate followed by hexane washing. Following purification, the sophorolipid  
153 material was subjected to alkaline hydrolysis by diluting in 5 M NaOH (*Merck*) and heating under reflux to 90°C  
154 for 10 min. The material was then acidified to pH 4 using 18.5% (w/v) HCl (*Merck*). ASL was then recovered via  
155 precipitation in a pentanol / hexane medium (*Merck*) at -18°C (Baccile et al. 2013). Following production and  
156 purification, SL congeners were identified by a UHPLC system with RS Diode Array detector (*ThermoFisher*  
157 *Scientific*) in conjunction with the amaZon SL dual funnel Ion Trap spectrometer LCMS system (*Bruker*). The  
158 percentage relative amounts of each congener were calculated and a ratio of ASL to LSL determined (Smyth et  
159 al., 2010).

160

161 Cell culture

162

163 Colorectal cancer cell lines HT29 (ATCC® HTB-38), HT115 (ECACC 85061104), HCT116 (ATCC® CCL-247),  
164 and Caco2 (ATCC® HTB-37) as well as colonic epithelium CCD-841-CoN (ATCC® CRL-1790) cell lines were  
165 used in this study. Cell lines were maintained in DMEM low glucose media or MEM low glucose media  
166 (*ThermoFisher Scientific*) supplemented with 10% (v/v) foetal bovine serum (*ThermoFisher Scientific*). All  
167 cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

168

169 Cell viability assay

170

171 A total of 1 x 10<sup>4</sup> cells per well were seeded in to a 96 well plate (*Sarstedt*) and allowed to attach overnight before  
172 being serum starved for 24 hours. Cells were then treated with concentrations of ASL ranging between 0.001 µg  
173 mL<sup>-1</sup> – 100 µg mL<sup>-1</sup> or vehicle-only control (PBS) for 24 hours. Subsequently, 10 µL of a 25 mg mL<sup>-1</sup> solution of  
174 MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) (*Merck*) was added to each well and the

175 plate further incubated for 1 hour at 37°C. Formazan crystals were solubilized with 100  $\mu$ L of DMSO (*Merck*).  
176 Absorbance at 570 nm was measured using a FLUOstar Omega microplate reader (*BMG-LABTECH*).  
177 Experiments were repeated three times with six internal replicates per treatment group. Data are representative of  
178 the three independent repeats and presented as mean percentage reduction in absorbance in comparison to vehicle-  
179 only controls  $\pm$  standard deviation (SD).

180

181 Visualisation of morphological changes induced by ASL treatment

182

183 A total of  $1 \times 10^4$  cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight  
184 forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 20 and 70  $\mu$ g mL<sup>-1</sup>  
185 of ASL or vehicle-only control (PBS) for 24 hours. The cells were subsequently imaged with an Axio Scope 1  
186 microscope (*Zeiss*) at 200  $\times$  magnification. Images obtained from three random fields were selected and assessed  
187 for morphological changes by comparing each cell line to the vehicle-only control, looking for changes in cell  
188 shape and confluency. Experiments were plated in triplicate and repeated three times.

189

190 Quantification of detached cells

191

192 A total of  $1 \times 10^4$  cells per well were seeded on to a 96 well plate (*Sarstedt*) and allowed to attach overnight  
193 forming a monolayer before being serum starved for 24 hours. Cells were then treated with both 40 and 70  $\mu$ g mL<sup>-1</sup>  
194 of ASL or vehicle-only control (PBS) for 24 hours. 10  $\mu$ g of supernatant was removed from each well, placed  
195 into a 1.5 mL microtube (*Sarstedt*). Cells were stained with 5  $\mu$ M of both Syto 9 and propidium iodide (*Merck*)  
196 by adding directly into the microtube and incubating at room temperature for 30 minutes. The cells were then  
197 centrifuged at 150  $\times g$  for 5 minutes and the supernatant was aspirated. The remaining cells pellet was washed  
198 using ice-cold PBS (pH 7.4) (*ThermoFisher Scientific*) and spun onto a microscope slide using a Shandon  
199 cytocentrifuge (*ThermoFisher Scientific*) for five minutes at 176  $\times g$ . Slides were subsequently imaged with a  
200 Axio Scope 1 microscope (*Zeiss*) at 400  $\times$  magnification. Following staining with Syto9 and propidium iodide,  
201 fluorescent staining was present in nucleus therefore live cells appeared green while dead cells fluoresced red  
202 (Altman et al. 1993) and as such cells were counted. A minimum of 1 cell and a maximum of 250 cells were  
203 counted per field of view. Experiments were plated in triplicate and repeated three times, data set shown as mean  
204  $\pm$  Standard Error of the Mean (SEM).



205

206 Acridine orange / ethidium bromide staining and quantification

207

208 To determine the mechanism of cell death induced by addition of ASL, cells were stained *in situ* with acridine  
209 orange at 10 mg mL<sup>-1</sup> (*Merck*) and ethidium bromide 1 mg mL<sup>-1</sup> (*Merck*). Morphological changes were assessed  
210 by fluorescence microscopy. For assessment of apoptosis, a total of 3 × 10<sup>4</sup> cells were seeded onto a 10 mm  
211 coverslip (*Agar Scientific*) placed within a 6 well plate and the cells were incubated overnight to form a confluent  
212 monolayer. Following serum starvation for 24 hours; ASL (at a concentration of either 20 µg mL<sup>-1</sup> or 70 µg mL<sup>-1</sup>)  
213 <sup>1</sup>), vehicle control (PBS), or 5 µM of etoposide (apoptotic control) (*Merck*) was added and the plate incubated for  
214 a further 24 hours. To determine the number of live cells remaining on the coverslip, the samples were washed  
215 three times with ice-cold phosphate buffered saline at pH7.4 (*ThermoFisher Scientific*), followed by incubation  
216 with 10 µL of a solution of acridine orange / ethidium bromide with a volumetric ratio of 1:1 for 5 minutes.  
217 Finally, the cells were washed again three times with ice-cold PBS at pH7.4 (*ThermoFisher Scientific*). Cells were  
218 subsequently imaged with a Axio Scope 1 fluorescence microscope (*Zeiss*) at 400 × magnification. A total of 300  
219 attached cells/coverslip were morphologically identified and counted as necrotic (red/orange nuclei), apoptotic  
220 (green condensed or fragmented nuclei) or live (green non-condensed ovoid or rounded nuclei). The operator was  
221 blinded to the experimental groups and random fields were selected. Each experiment was replicated three times  
222 with six internal repeats per group. Data are representative of three independent repeats and presented as mean ±  
223 SEM.

224

225 Scratch assay

226

227 For wound healing scratch assays, 1.6 × 10<sup>6</sup> cells were plated in each well of a 6 well plate (*Sarstedt*) and allowed  
228 to attach overnight. Cells were serum starved for 24 hours and treated with 5 µg mL<sup>-1</sup> of mitomycin C (*Merck*)  
229 for 2hours prior to the scratch to inhibit proliferation. The “wound” was made by scratching a line in the centre  
230 of the confluent monolayer using a sterile toothpick. Cells were rinsed very gently three times with PBS at pH7.4  
231 (*ThermoFisher Scientific*) and cultivated in serum free media supplemented with PBS vehicle-control or 10 - 20  
232 µg mL<sup>-1</sup> ASL for up to 72 hours. The cell was imaged at various time points using a ELWD TI SCP microscope  
233 (*Nikon Europe B. V.*) at 100 × magnification. To quantify migration of cells into the scratch wound, the area of  
234 the gap was measured using ImageJ software (Schneider et al. 2012). After 72 hours, the area of the remaining

235 gap was measured and the difference between initial and final areas calculated. Experiments were repeated three  
236 times using triplicate technical replicates per experimental group. Data are representative of the three independent  
237 repeats and presented as mean  $\pm$  SEM.

238

239 Chemotactic cell migration assays

240

241 Chemotactic cell migration assays were carried out in a modified Boyden chamber, based on a previously  
242 described protocol (Chen 2005).  $1 \times 10^4$  cells per well of CCD-841-CoN, HT29 or HT115 CRC cells were placed  
243 in the upper compartment of a 96- well FluoroBlok transwell inserts (*Analab*) which contained 8  $\mu$ m pore size  
244 polyethylene terephthalate filters (*Analab*) and the cells were allowed to attach overnight. A final concentration  
245 of 0, 10 or 50  $\mu$ g mL<sup>-1</sup> of ASL in serum free media was added to the upper chamber, while appropriate culture  
246 media supplemented with 10% FBS was added (as a chemoattractant) to the bottom chamber. Cells were left to  
247 migrate overnight in an incubator at 37°C and at 5% CO<sub>2</sub>. Following incubation, all media in the upper chamber  
248 was removed and all cells that failed to migrate were removed using a sterile cotton swab. Cells that had migrated  
249 through to the lower surface of the filter insert were stained with a 0.1% (w/v) solution of crystal violet (*Merck*)  
250 made up in 25% (v/v) methanol (*Merck*). A total of three random areas were chosen and a minimum of 1 cell and  
251 a maximum of 300 cells stained with crystal violet were counted under a Axio Scope 1 light microscope (*Zeiss*)  
252 at 400  $\times$  magnification. Migration rates were expressed as total percentage of the control. Data are representative  
253 of the three independent repeats and presented as mean  $\pm$  SEM.

254

255 Animal model

256

257 Prior to the commencement of the study all animal procedures were approved by both the animal care and ethics  
258 committee at Ulster University and the UK Home Office. Additionally, all animal procedures were carried out by  
259 licensed personnel in accordance with both local animal welfare committee (Ulster University) and national (UK  
260 Home Office) guidelines (Workman et al. 2010). For breeding purposes male *Apc*<sup>min+/-</sup> mice were housed together  
261 with female wild type (*wt*) mice and all the animals subjected to a 12/12 light cycle, with food and water being  
262 available *ad libitum*. Mice were genotyped as described previously in Callaghan *et al.*, (2016), with both  
263 heterozygous *Apc*<sup>min+/-</sup> and *wt* mice (male and female) used in experiments. During the study mice were monitored  
264 daily for grooming activity, general behaviour, activity levels, food and water intake and general wellbeing. Mice

265 assessed as exhibiting signs of distress or discomfort were immediately removed from the study and euthanized  
266 via CO<sub>2</sub> asphyxiation.

267

268 ASL dosing

269

270 At five weeks of age, both *wt* littermate and *Apc*<sup>min/+</sup> mice were treated via oral gavage every other day with either  
271 vehicle-only or a solution containing 50 mg kg<sup>-1</sup> (body weight) of ASL suspended in saline for 70 days. During  
272 treatment, body weights, general health and behaviour were monitored bi-weekly. Food and water were weighed  
273 on a weekly basis to determine the effect of ASL treatment on eating/drinking habits. A cut-off point for body  
274 weight reduction of 10% was applied and mice reaching this point were euthanized via CO<sub>2</sub> euthanasia.

275

276 Tissue collection and assessment

277

278 At the end of the experimental protocol, mice were euthanized with an overdose of pentobarbitone (200 mg kg<sup>-1</sup>  
279 given IP). A cardiac puncture was performed to collect blood that was stored in EDTA tubes (*Aquilant Scientific*).  
280 An additional blood sample was taken for haematocrit level estimation. For this a blood capillary tube was filled  
281 with blood, sealed, and centrifuged (Microcentrifuge with Hematocrit Rotor; *Cole-Parmer*) at 13,000 × *g* for 5  
282 minutes. Haematocrit levels were calculated using the following calculation: height of RBC/total height of all the  
283 components × 100. Internal organs including the intestinal tract, colon, spleen, heart, liver, kidneys and lungs  
284 were removed, weighed and immerse fixed in 10% buffered formal saline at pH7.4 (*ThermoFisher Scientific*).  
285 The intestinal tracts were divided into 3 sections according to the description provided by Casteleyn *et al.*, (2010).  
286 After identification of the specific intestinal regions, samples were bisected longitudinally, and the total number  
287 of polyps was recorded as well as their diameters measured with callipers (Casteleyn *et al.* 2010). The specimens  
288 were then cut into ~2 cm strips and placed in cassettes prior to standard wax embedding. To assess qualitative  
289 histopathological changes in the intestines and spleen, tissues were cut into 5 μm sections using a Shandon Finesse  
290 325 Microtome (*ThermoFisher Scientific*) placed on glass slides, cleared with xylene, dehydrated in descending  
291 grades of ethanol and subsequently stained with Mayer's haematoxylin and eosin (*Merck*). Stained sections were  
292 examined with a Axio Scope 1 light microscope (*Zeiss*) at a range of objective magnifications.

293

294 Statistical analyses

295

296 Statistical analysis of cell viability data was determined by two-way ANOVA followed by both Tukey's and  
297 Dunnett's post hoc testing. Analysis of live/ dead cell counts was determined by two-way ANOVA followed by  
298 Šidák's post hoc testing. Analysis of remaining *in vitro* data was determined by two-way ANOVA followed by  
299 both Dunnett's post hoc testing. Comparisons between *in vivo* groups was assessed using a students' t-test. A  
300 value of  $p \leq 0.05$  was considered statistically significant. All statistical analysis was carried out with the aid of  
301 Prism Version 9.3.1 (350) (*GraphPad Software*).

302

### 303 **Results**

304

#### 305 Production and purification of ASL

306

307 A purified form of diacetylated ASL was produced for use in in this study from a commercially available SL  
308 formulation using methodologies outlined by Baccile *et al.* (2013). Following the recommendation of Twigg *et*  
309 *al.* 2021, the purified sample was analyzed via HPLC-ELSD and was found to be composed of 94% nonacetylated  
310 ASL. A breakdown of the various nonacetylated ASL congeners present in the samples can be seen in Table S1.  
311 The most abundant congener present in the sample was acidic C18:1s (75% relative abundance). The acidic C18:1t  
312 and C18:2t congeners represented 10% each, while all other ASL congeners were limited to less than 2% relative  
313 abundance (Twigg *et al.* 2021).

314

315 ASL have a selective effect on colorectal cancer cell viability.

316

317 Several colorectal tumour cell lines (HT29, HT115, HCT116 and Caco2), in addition to a colonic epithelial cell  
318 line (CCD-841-CoN) were treated with ASL to assess the effect on cell viability. ASL at concentrations up to 100  
319  $\mu\text{g mL}^{-1}$  effected no significant decrease in the viability of the CCD-841-CoN cell line after 24 hours of treatment  
320 in comparison to cultures treated with a vehicle-only control (PBS) (Fig. 1a). However, 24 hours of treatment with  
321 ASL concentrations equal to or above 1  $\mu\text{g mL}^{-1}$  resulted in significantly reduced viability in the HT115 cell line  
322 in comparison to vehicle-only controls ( $p = 0.0201$ ) (Fig. 1a). In HT29, Caco2 and HCT116 cell lines, treatment  
323 with ASL concentrations equal to or above 10  $\mu\text{g mL}^{-1}$  resulted in a significant reduction in cell viability in  
324 comparison with cells treated with vehicle-only controls ( $p = < 0.0001$ ,  $< 0.0001$ , and 0.0099 respectively) (Fig.

325 1a). The viability of colorectal tumour cell lines HT29, HT115 and Caco2 were significantly reduced when  
326 compared to the colonic epithelial cell line CCD-841-CoN when treated with ASL concentrations equal to or  
327 above  $1 \mu\text{g mL}^{-1}$  ( $p = 0.0146, < 0.0001, \text{ and } 0.0017$  respectively). For cell line HCT116 a significant reduction in  
328 viability when compared to CCD-841-CoN was observed when cells were treated with ASL concentrations equal  
329 to or above  $10 \mu\text{g mL}^{-1}$  ( $p = 0.0046$ ) (Fig. 1a).

330

331 ASL induces cell rounding, detachment and cell death in colorectal cancer cells

332

333 To observe potential effects on cell morphology, colorectal cancer cell lines (HT29, HT115, HCT116 and Caco2)  
334 and the colonic epithelial cell line (CCD-841-CoN) were treated with ASL and assessed by microscopic  
335 examination. CCD-841-CoN cells treated with a vehicle-only control (PBS) grew as a confluent monolayer with  
336 a bipolar morphology (Fig. S2). Following treatment with either  $20$  or  $70 \mu\text{g mL}^{-1}$  ASL no morphological  
337 differences of cells in the monolayer were observed when compared to vehicle-only treated CCD-841-CoN cells  
338 (Fig. S2). HT29, HT115, HCT116 and Caco2 treated with the vehicle-only control displayed a densely packed,  
339 cobblestone-like monolayer morphology (Fig. S2). When treated with ASL, disruption to the monolayer and  
340 detachment of HT-29, HT115, HCT116 and Caco-2 cell lines was observed. Partially detached and rounded cells  
341 were conspicuous within HT29, HT115 and Caco2 cell lines treated with  $20 \mu\text{g mL}^{-1}$  (Fig. S2). HT29, HT115,  
342 HCT116 and Caco2 cells exposed to  $70 \mu\text{g mL}^{-1}$  ASL, demonstrated extensive monolayer disruption with  
343 distinctive cell free areas within the cultures (Fig. S2).

344 As cellular detachment was observed in all tumour cell cultures, cells were isolated from the supernatant  
345 and stained with syto9 and propidium iodine to determine if detachment resulted in cell death. Only a small  
346 number of CD-841-CoN cells treated with either concentration of ASL were observed to detach and there was no  
347 significant difference in the proportion of these detached cells that were either alive or dead (Fig. 1b). In contrast  
348 the number of detached HT29, HT115, HCT116 and Caco2 cells was higher when treated with either  
349 concentration of ASL when compared to vehicle-only treated cells, and there was a significant increase in the  
350 proportion of these detached cells that were found to be dead (Fig. 1b).

351

352 ASL induces cell death *in vitro* via both apoptotic and necrotic pathways.

353

354 As treatment with ASL resulted in a significant loss of cell viability, and induced detachment and cell death in  
355 colorectal cancer cell lines, the mechanism of cell death was investigated. Colorectal cancer (HT29, HT115 and  
356 HCT116 and Caco2) and colonic epithelial (CCD-841-CoN) cells lines were treated with ASL and stained with  
357 acridine orange and ethidium bromide to determine whether cell death was morphologically identifiable as a result  
358 of apoptosis or necrosis. Treatment of CCD-841-CoN cells with 20  $\mu\text{g mL}^{-1}$  of ASL did not result in a significant  
359 increase in the numbers of either apoptotic or necrotic cells. However, treatment with 70  $\mu\text{g mL}^{-1}$  ASL resulted  
360 in 10% cell death within the culture that was identified morphologically as apoptosis ( $p < 0.05$ ) (Fig. 1c). A dose-  
361 dependent increase in cell death in all four human colorectal cancer cell lines was observed following ASL  
362 treatment (Fig. 1C). In HT29, HT115 and HCT116 cell lines treated with 20  $\mu\text{g mL}^{-1}$  ASL, there were equivalent  
363 numbers of apoptotic and necrotic cells. However, there was a higher number of apoptotic cells in Caco2 cells  
364 treated with 20  $\mu\text{g mL}^{-1}$  ASL ( $p < 0.01$ ) (Fig. 1c). Exposure of cultures to 70  $\mu\text{g mL}^{-1}$  ASL, resulted in a markedly  
365 reduced number of adherent colorectal cancer cells available for quantification. HT29 and HT115 cultures treated  
366 with 70  $\mu\text{g mL}^{-1}$  showed a significant increase in the numbers of apoptotic and necrotic cells ( $p < 0.001$ ,  $p < 0.01$   
367 respectively) while HCT116 cells were predominantly necrotic ( $p < 0.0001$ ), and Caco-2 cells had a higher number  
368 of apoptotic cells ( $p < 0.001$ ) compared to vehicle-only controls (Fig. 1c).

369

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

371

372 To assess the effect of ASL on cell migration, a scratch was made across a monolayer of colorectal cell lines HT29  
373 and HCT115, and the colonic epithelial cell line CCD-841-CoN. Treatment with 10  $\mu\text{g mL}^{-1}$  ASL had no  
374 significant effect on CCD-841-CoN cell migration, with 90% of the total scratch area being covered after 72 hours  
375 (Fig. 2a). In contrast, treatment with 10  $\mu\text{g mL}^{-1}$  ASL resulted in a highly significant decrease in the proportion  
376 of the total scratch area covered in HT-29 (10%;  $p < 0.0001$ ) and HT115 cells (22%;  $p < 0.0001$ ) after 72 hours  
377 (Fig. 2a). To test the effect ASL had on chemotaxis, the same cell lines were plated in the upper portion of a  
378 Boyden chamber and the number of cells migrating in response to FCS were counted after 24 hours. Media  
379 supplemented with either 10 or 50  $\mu\text{g mL}^{-1}$  ASL did not significantly affect the migration of CCD-841-CoN cells.  
380 The addition of 10  $\mu\text{g mL}^{-1}$  ASL to media did however result in a significant reduction in migration of HT29  
381 (33%) and HT115 (29%) compared to control values ( $p < 0.001$ ) (Fig. 2b). The addition of 50  $\mu\text{g mL}^{-1}$  ASL  
382 reduced migration of HT29 and HT115 to 8.3% and 10.4% of control values respectively ( $p < 0.0001$ ) (Fig. 2b).

383

384 *In vivo* experiments: *wt* and *Apc*<sup>min+/-</sup> mice tolerate the oral administration of ASL

385

386 To determine palatability and potential toxicity of ASL, a pilot study was performed with both *wt* and *Apc*<sup>min+/-</sup>  
387 mice which were fed either a vehicle-only control solution consisting of 10% sucrose water or vehicle containing  
388 either 0.5, 5 or 50 mg kg<sup>-1</sup> ASL for a period of 5 weeks (n = 3/group). Both *wt* and *Apc*<sup>min+/-</sup> mice gained weight  
389 at a similar rate and there was no change in food or water intake between any of the treatment groups (Fig. S3)  
390 On completion of this oral feeding tolerance study, the major organs (liver, stomach, kidneys, lungs, heart, spleen  
391 and pancreas) were removed, weighed and a gross inspection of morphology was carried out. There were no  
392 significant differences in organ weights (Table S2) or gross organ morphology observed between mice fed on the  
393 vehicle only control diet or on ASL.

394

395 ASL reduces polyp-associated bleeding and increases haematocrit levels but does not affect tumour size or  
396 numbers in *Apc*<sup>min+/-</sup> mice.

397

398 The gross morphological appearances of unfixed, flat-mounted ilea from *wt* mice treated with either vehicle-only  
399 control or 50 mg kg<sup>-1</sup> ASL was characterised by a flattened, uniformly smooth mucous epithelium with prominent  
400 patent blood vessels (Fig. 3a). In vehicle-only treated *Apc*<sup>min+/-</sup> mice, there was clear evidence of polyp-associated  
401 bleeding within the ileal segment (Fig. 3a). In contrast, *Apc*<sup>min+/-</sup> mice treated with 50 mg kg<sup>-1</sup> ASL for 70 days,  
402 showed little evidence of bleeding from these intestinal polyps (Fig. 3a). However, the number of intestinal polyps  
403 in *Apc*<sup>min+/-</sup> mice was not significantly different following treatment with 50 mg kg<sup>-1</sup> ASL for 70 days in  
404 comparison the vehicle-only control treated cohort (vehicle = 48 ± 2 vs ASL = 45 ± 4; *p* < 0.1) (Fig. 3b). ASL  
405 treatments also had no effect on the modal size distribution of the polyps in comparison to vehicle-only treatment  
406 (vehicle- 4 mm vs ASL 4 mm; *p* > 0.05) (Fig. 3c).

407 No significant difference in hematocrit level were observed in *wt* mice fed with either vehicle-only  
408 control or 50 mg kg<sup>-1</sup> ASL for 35 or 70 days (*p* = 0.8132 and *p* = 0.9685 respectively) (Fig. 3d and Fig. 3e).  
409 Hematocrit levels in *wt* mice were also significantly higher than those of *Apc*<sup>min+/-</sup> mice, irrespective of treatment  
410 with either vehicle-only control or ASL (*p* = > 0.05) (Fig. 3d and Fig. 3e). After 35 days feeding with 50 mg kg<sup>-1</sup>  
411 ASL, no significant differences in haematocrit levels were observed in *Apc*<sup>min+/-</sup> mice compared to those fed with  
412 vehicle-only control (vehicle-only = 41.2 ± 0.8 vs ASL = 40.0 ± 1.1; *p* = 0.9906) (Fig. 3d). However, following  
413 70 days of administration with 50 mg kg<sup>-1</sup> ASL, haematocrit levels were found to be significantly higher in

414  $Apc^{min/+}$  mice fed in comparison to those fed with the vehicle-only control (vehicle-only =  $36.0 \pm 0.9$  vs ASL =  
415  $42 \pm 1.9$ ;  $p = 0.012$ ) (Fig. 3e).

416

417 ASL effects the splenic weight, proportion of spleen red pulp in  $Apc^{min/+}$  mice

418

419 The spleens from vehicle-only control fed  $Apc^{min/+}$  mice were significantly heavier than their *wt* littermates ( $0.58$   
420  $\pm 0.2g$  vs  $0.15g \pm 0.5g$ ;  $p < 0.0001$ ). A dose of  $50 \text{ mg kg}^{-1}$  ASL had no effect on splenic weight in *wt* mice after  
421 70 days of treatment. However, administration of  $50 \text{ mg kg}^{-1}$  ASL for 70 days to  $Apc^{min/+}$  mice resulted in a  
422 statistically significant decrease in splenic weight ( $0.58 \pm 0.2 \text{ g}$  vs  $0.40 \pm 0.4g$ ;  $p < 0.001$ ) (Fig. 4a). Following  
423 treatment of the  $Apc^{min/+}$  mice with  $50 \text{ mg kg}^{-1}$  ASL there was a significant reduction in the proportion of red pulp  
424 as compared with vehicle-only controls ( $62 \pm 3.2$  vs  $48 \pm 1.4$ ;  $p < 0.001$ ), no significant difference was observed  
425 in the *wt* mice (Fig. 4b). Histological examination of sections from *wt* mouse spleen revealed conspicuous and  
426 intensely basophilic areas of white pulp; these were separated by less dense regions of red pulp; areas that are  
427 responsible for removal of old or damaged erythrocytes. In vehicle-only control treated  $Apc^{min/+}$  mice there was  
428 obvious clumping of the red pulp, and the proportion of this tissue was significantly increased when compared to  
429 *wt* mice (Fig. 4c).  $Apc^{min/+}$  mice fed with ASL showed reduced clumping of red pulp and an increase in the white  
430 pulp regions, like that of the *wt* mice (Fig. 4c).

431

## 432 Discussion

433

434 The ability of chemotherapeutic agents to selectively target cancerous cells while sparing normal tissue is a highly  
435 desirable trait, as it may mitigate against the common side effects associated with these toxic therapeutics such as  
436 epithelial cell damage in the gastrointestinal tract, immunosuppression (in the bone marrow) and hair loss (Carey  
437 and Burish 1988; McQuade et al. 2014). Biosurfactant compounds such as sophorolipids are naturally produced  
438 agents that may possess the ability to differentially affect cancer and normal epithelial cells and as such their anti-  
439 cancer activities are a growing area of research. Here we investigated the *in vitro* effects of a 94% pure preparation  
440 of ASL on a non-transformed intestinal epithelial cell line (CCD-841-CoN) and 4 colorectal cancer cell lines  
441 (HT29, HT115, Caco2, and HCT116). Colonic epithelia (CCD-841-CoN) treated with ASL showed no sign of  
442 toxicity as evidenced by maintenance of their viability at doses ranging between  $0.001$  and  $100 \mu\text{g mL}^{-1}$ . However,  
443 at doses of  $20 \mu\text{g mL}^{-1}$  and above, ASL's potently reduced cell viability in all the colorectal cancer cell lines



444 examined. Although the cytotoxic potency of sophorolipid mixtures has been reported against cancer cell lines  
445 previously, (Chen et al. 2006b; Fu et al. 2008; Shao et al. 2012; Ribeiro et al. 2015; Callaghan et al. 2016; Adu et  
446 al. 2022), this is the first time an anti-cancer effect has been reported from a purified and well characterised ASL  
447 on a range of cell lines from the same tissue of origin (i.e. colorectal cancer cells).

448 Importantly, the ASL preparation utilised here exhibited a differential effect *in vitro* on non-transformed  
449 cell lines compared to CRC cells, with a dose-dependent cytotoxic response in all five CRC cell lines. A  
450 differential effect has been reported in previous studies where SL were cytotoxic to pancreatic, liver and melanoma  
451 cancer cell lines but with no demonstrated toxicity to non-transformed cell lines (Fu et al. 2008; Adu et al. 2022).  
452 However, Fu *et al.* 2008 utilised non-adherent circulating blood monocytes (PBMC) as a control for adherent  
453 transformed cells making conclusions from this study difficult to interpret. Therefore, other than Adu and  
454 colleagues work on melanoma cell lines, a directly comparable description of the specificity of a pure preparation  
455 of ASL that uses a normal adherent cell line from the same tissue of origin has not been carried out.

456 It has been hypothesised that SL can intercalate into the cytoskeleton of cells resulting in membrane  
457 disruption. Changes in cell morphology consistent with disruption of membrane cytoskeletal protein distribution  
458 have previously been shown to occur with SL doses as low as 30  $\mu\text{g mL}^{-1}$  in pancreatic H7402 and lung cancer  
459 A549 cell lines (Chen et al. 2006b). Our study demonstrated that a preparation dominated by a single congener of  
460 ASL at low concentrations (40  $\mu\text{g mL}^{-1}$ ) induced cell rounding, cytoplasmic condensation and cell detachment in  
461 all the CRC cancer cell lines tested, with the adenocarcinoma cell lines HT29 and Caco2 being most susceptible.  
462 At doses of 70 – 100  $\mu\text{g mL}^{-1}$ , ASL caused a significant increase in the numbers of detached cells with all cells in  
463 the supernatant from treated cultures showing morphological features consistent with apoptosis or necrosis.  
464 Similar results have been observed in HepG2 liver cancer cells treated with LSL preparations (Wang et al. 2021).

465 ASL's used at the doses described in our study, induced a reduction of tension at the interfacial region  
466 of the bilayer resulting in phospholipid dehydration which affected lipid stability and ultimately resulted in cell  
467 death (Maget-Dana et al. 1989; Shah et al. 2005; Ortiz et al. 2009). To assess cell death mechanisms caused by  
468 ASL's, ethidium bromide/acridine orange staining was carried out allowing the morphological identification of  
469 the type of cell death. We have conclusively demonstrated that ASL induces both apoptosis and necrosis in these  
470 cancer cell lines in a dose-dependent manner. The ability of ASL to induce either apoptosis or necrosis may be  
471 cell-line specific, as high proportions of apoptotic cells have been reported following the addition of diacetylated  
472 LSL to liver (H7402) and lung cancer (H7402) cell cultures, while necrosis is primarily observed in a pancreatic

473 carcinoma cell line (HPAC) treated with sophorolipid mixtures, LSL or methyl ester derivative SL (Shao et al.  
474 2012).

475 An important characteristic of malignant growth is the ability of tumour cells to leave their restricted  
476 compartment and gain access to blood vessels to initiate the first phase of metastasis (Hanahan and Weinberg  
477 2011). The movement of cells across tissues therefore plays an important role in this progression thus highlighting  
478 the need for an agent that can counteract the migratory and diapedesis properties of colorectal cancer cells  
479 (Dianzani et al. 2014). It has been postulated that the amphiphilic properties of sophorolipids permit their  
480 incorporation into the mammalian cellular membrane disrupting cellular functions such as proliferation and  
481 migration (Zhao et al. 2013; Haque et al. 2021; Adu et al. 2022). We assessed the ability of ASL's to inhibit  
482 migration of the CRC cell lines HT29 and HT115. At a low dose ( $10 \mu\text{g mL}^{-1}$ ) of ASL there was no effect on  
483 normal colonic epithelial cells (CCD-841-CoN) and scratch coverage after 72 hours. However, the same dose of  
484 ASL applied to colorectal cancer cell lines HT29 and HT115 cultures reduced the total percentage area of scratch  
485 covered to between 12-25%. An inhibition of migration induced by sophorolipid (as measured in the scratch assay)  
486 has been documented. In 2015, Riberiro and co-workers showed that  $5 \mu\text{g mL}^{-1}$  of a 93% pure, diacetylated LSL  
487 resulted in significantly reduced migration of MDA-MB-231 breast cancer cells (Ribeiro et al. 2015), although to  
488 our knowledge no reports are available for a study that solely utilises purified ASL. Adu *et al.* 2022 showed that  
489 the migration of a melanoma cell line SK-MEL-28 was significantly reduced when treated with LSL and ASL  
490 preparations in comparison with cells treated with a vehicle-only control, and immortalized keratinocytes (HaCaT)  
491 treated with the same sophorolipid preparations (Adu et al. 2022). The use of both migration and invasion assays  
492 incorporating the Boyden chamber are widely used to test candidate chemotherapeutics with the HT29 and HT115  
493 cell lines commonly employed to test anti-invasive properties of potential chemotherapeutics *in vitro* (Li and Zhu  
494 1999). Currently the only studies investigating the potential anti-metastatic properties of biosurfactants are limited  
495 to the inhibitory effects on breast cancer cell lines MCF and MDA-MB-231 *in vitro* invasion mediated by the  
496 lipopeptide Surfactin produced by *Bacillus subtilis* (Park et al. 2013). Addition of  $10 \mu\text{M}$  (equivalent to approx.  
497  $660 \mu\text{g mL}^{-1}$  of ASL in this study) of Surfactin had the ability to reduce the migration of MCF-7 and MDA-MB-  
498 231 through an extracellular matrix by 68% and 84% respectively (Park et al. 2013). The Surfactin used by Park  
499 *et al.* (2013) also reduced the colony forming ability of both cell lines by 70% and 61%. To investigate the  
500 migration inhibitory properties of ASL, HT29 and HT115 cells were plated on a porous membrane and allowed  
501 to migrate through a septum in response to a FBS stimulus in the chamber below. ASL had no effect on the  
502 migration of normal colonic CCD-841-CoN cells, however following treatment with  $10 \mu\text{g mL}^{-1}$  and  $50 \mu\text{g mL}^{-1}$

503 ASL, migration was significantly decreased in HT29 cell lines by 65% and 72% respectively, and 86% and 81%  
504 in HT115 cell lines.

505 The anti-tumour activities of sophorolipid preparations *in vivo* are unclear. In a study by Li and co-  
506 workers, an intragastric administered mixture composed of 6 di-acetylated LSL congeners was shown to reduce  
507 the size of tumours in a murine HeLa xenograft model (Li et al. 2017). Whereas a purified form of LSL  
508 exaggerated the growth of neoplasm along the intestinal tract and increased intestinal blood loss in the  $Apc^{min/+}$ -  
509 mouse (Callaghan et al. 2016). In this study, we use the  $Apc^{min/+}$  mouse model of FAP, to evaluate the  
510 chemotherapeutic potential of purified ASL in the treatment of solid neoplasms.  $Apc^{min/+}$  mice develop intestinal  
511 adenomatous neoplasms (polyps), and animals typically present with enlarged spleens and reduced haematocrit  
512 by 4 months of age (Yekkala and Baudino 2007). This is an acute model with a life span of <150 days, where the  
513 primary cause of death is not directly attributable to the development of numerous polyps but rather as a result of  
514 extensive intestinal bleeding and anaemia (Hinoi et al. 2007). When administered orally, topically or via *i.v.*  
515 injection sophorolipid mixtures are well tolerated (Ikeda et al. 1986). Similarly, in our study, both *wt* and  $Apc^{min/+}$ -  
516 mice tolerated the oral administration of ASL with no measurable gross anatomical or behavioural differences  
517 noted. Post-mortem analysis also revealed no effects on gross measures of peritoneal organs (size and dimensions)  
518 after ASL treatment. However, oral feeding of ASL to  $Apc^{min/+}$  for 70 days resulted in a reduction of spleen size  
519 and a significant increase in haematocrit, consistent with decreased intestinal bleeding and improvement in the  
520 associated anaemia characteristic of this model (Perkins et al. 2002; Hinoi et al. 2007). This is a potentially  
521 significant finding, as rectal bleeding and anaemia are reported in over 30% of CRC patients and it is a contributing  
522 factor in reduced lifespan in both humans as well as  $Apc^{min/+}$  mice (Ronnekleiv-Kelly and Kennedy 2011). In  
523 humans, laser ablation encourages coagulation of tumours demonstrating significant blood loss and this technique  
524 has shown to be effective after 2 - 5 treatments with a success rate of 80% (Kimmey 2004). However recurrent  
525 bleeding episodes result in surgical intervention in 2 - 15% of patients (Rao et al. 2005). The oral administration  
526 of a well-tolerated non-toxic, pro-thrombotic agents to reduce intestinal blood loss in patients with haemorrhagic  
527 colorectal tumours may be a useful addition to the therapeutic treatment of these conditions.

528 In conclusion, the purified ASL mixture we utilised in this study differentially affects non-transformed  
529 in comparison to colorectal cancer cell lines, resulting in a significant and dose-dependent decrease in their  
530 viability, migration, and anchorage-independent growth characteristics. While ASL does not change either the  
531 size or number of intestinal polyps in  $Apc^{min/+}$  mice, both spleen size and tumour-associated bleeding was reduced.

532 This warrants further investigation of this orally available biosurfactant as a chemotherapeutic for delaying disease  
533 progression in pre-cancerous colorectal neoplasms.

534

#### 535 **Author Contributions**

536

537 BC and MST contributed equally to this work. INAVB, RM, CM and IMB conceived and designed the research.

538 BC and NB conducted experiments. BC and MST analysed data and wrote the manuscript. All authors read and

539 approved the manuscript.

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722

### 723 **Fig. Legends**

724

725 **Fig. 1.** Treatment with ASL results in a detrimental effect on colorectal cancer cell lines *in vitro*. Compared to  
726 normal colonic epithelial cells, the viability of four colorectal cancer cell lines was significantly reduced when  
727 treated with ASL (a). Treatment with ASL also resulted in increased cell detachment in colorectal cancer cell lines  
728 compared to normal colonic epithelial cells (b). Colorectal cancer cell lines treated with either 20 or 70  $\mu\text{g mL}^{-1}$   
729 ASL showed indications of both apoptosis and necrosis driven cell death (c).

730

731 **Fig. 2.** Treatment with ASL significantly reduces cell migration in colorectal cancer cell lines. In a scratch assay  
732 ASL treated colorectal cancer cells showed significantly less migration 72 h post treatment than these treated with  
733 vehicle-only controls (a). Cell migration across a Boyden chamber in response to a stimulus was significantly  
734 reduced in colorectal cancer cells treated with ASL in comparison vehicle-only control treated cells (b). Two-way  
735 ANOVA (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

736

737 **Fig. 3.** An ASL supplemented diet reduced intestinal bleed in a mouse mode of colorectal cancer. No  
738 morphological differences were observed in ileal sections of either wt or *Apc<sup>min/+</sup>* mice fed with either vehicle-  
739 only controls or ASL, however vehicle-only control fed *Apc<sup>min/+</sup>* mice showed evidence of intestinal bleeding  
740 from polyps (arrows), whereas ASL fed *Apc<sup>min/+</sup>* mice showed no evidence of intestinal bleeding (a). No  
741 significant difference in intestinal polyp number (b) or polyp sizes (c) was observed in *Apc<sup>min/+</sup>* mice fed on either

742 ASL or vehicle-only control. Following 35 days no significant difference was observed in haematocrit in  $Apc^{min+/-}$   
743 mice fed with ASL or vehicle-only control (**d**) however, after 70 days a significant increase in haematocrit in  
744  $Apc^{min+/-}$  mice fed with ASL was observed compared to those fed with vehicle-only control (**e**). One-way ANOVA,  
745  $p$  values displayed on graphs.

746

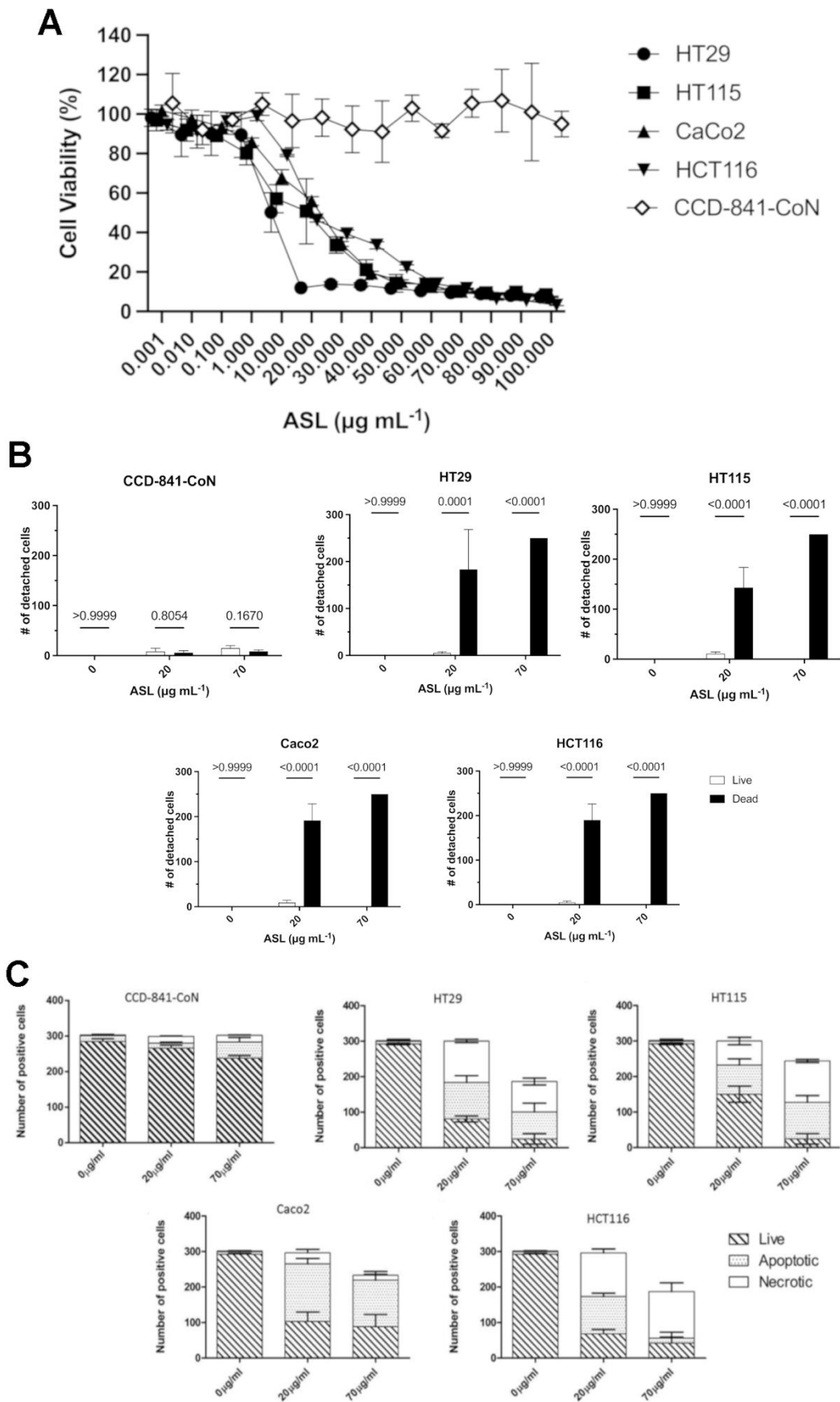
747 **Fig. 4.** Following 70 days, both splenic weight (**a**) and percentage of red pulp regions (**b**) in  $Apc^{min+/-}$  mice fed  
748 with ASL were significantly reduced in comparison to those fed with vehicle-only control ( $p < 0.001$  and  $p < 0.05$   
749 respectively).  $Apc^{min+/-}$  mice fed with vehicle-only control had increased red pulp with a loss reticular structure  
750 compared to wild-type mice. Splenic morphology (particularly in white pulp) after feeding  $Apc^{min+/-}$  mice with  
751 ASL more closely resembled that seen in wild-type mice (**c**).

752

753

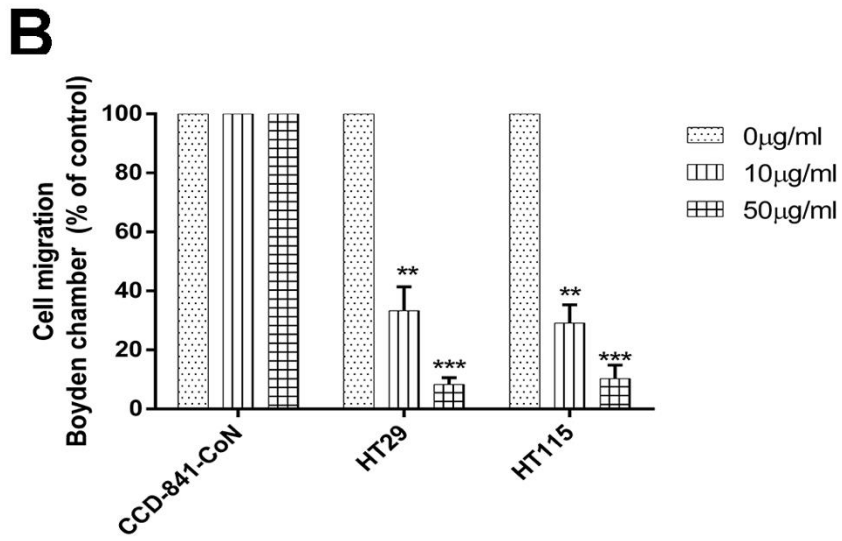
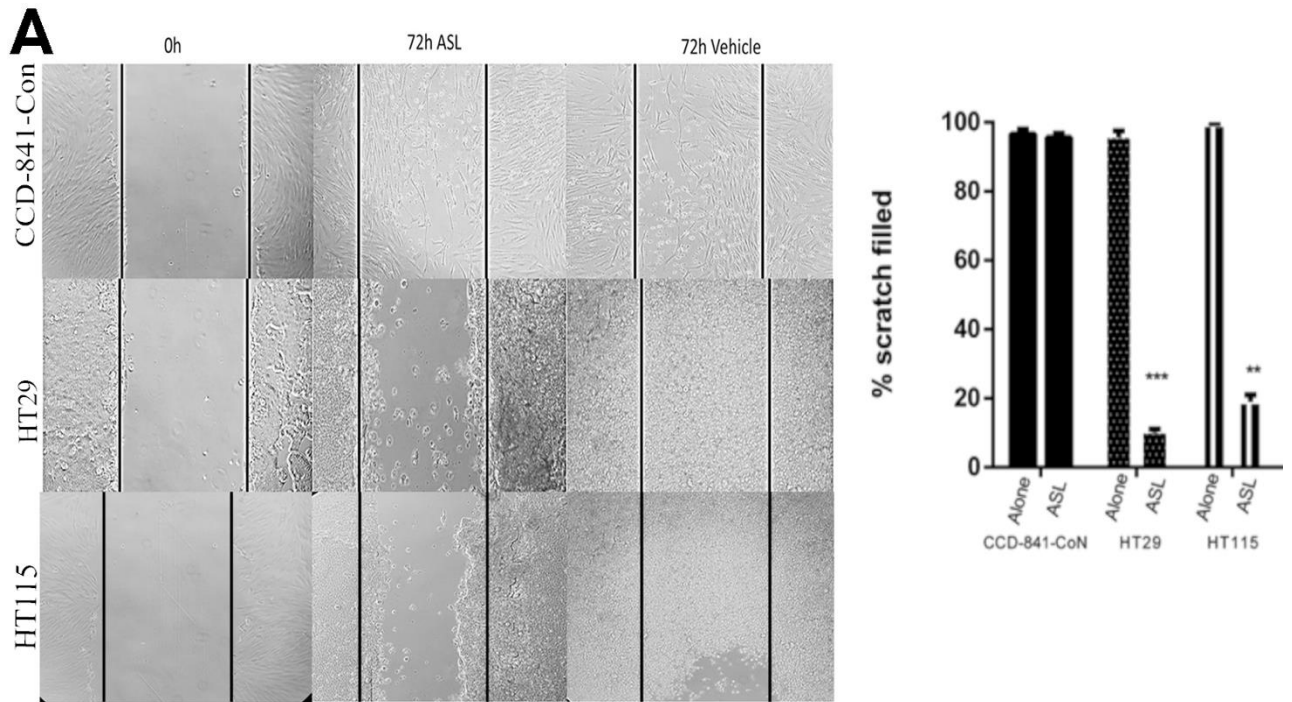
754

755 Fig. 1.



756

757 **Fig. 2.**



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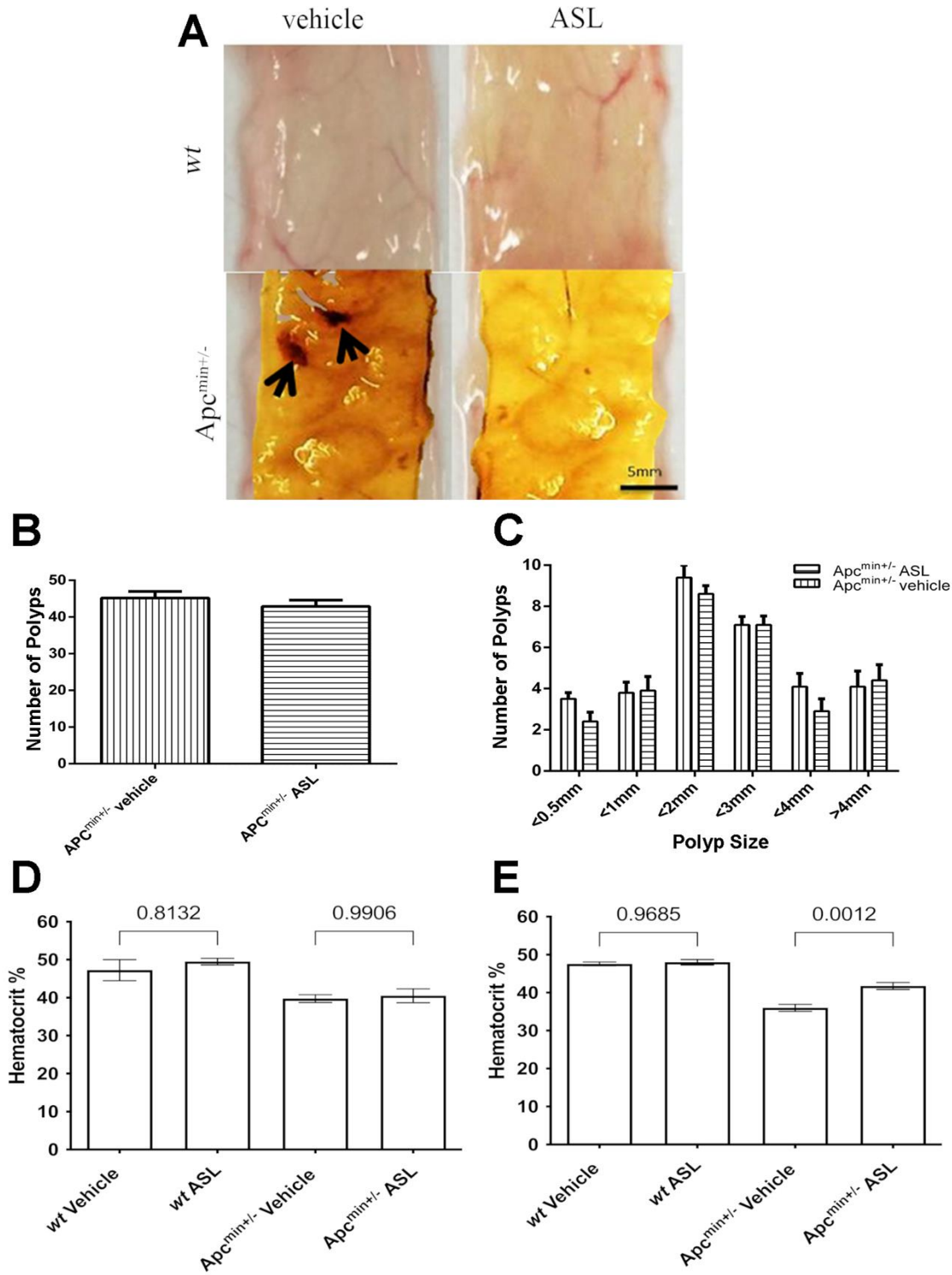
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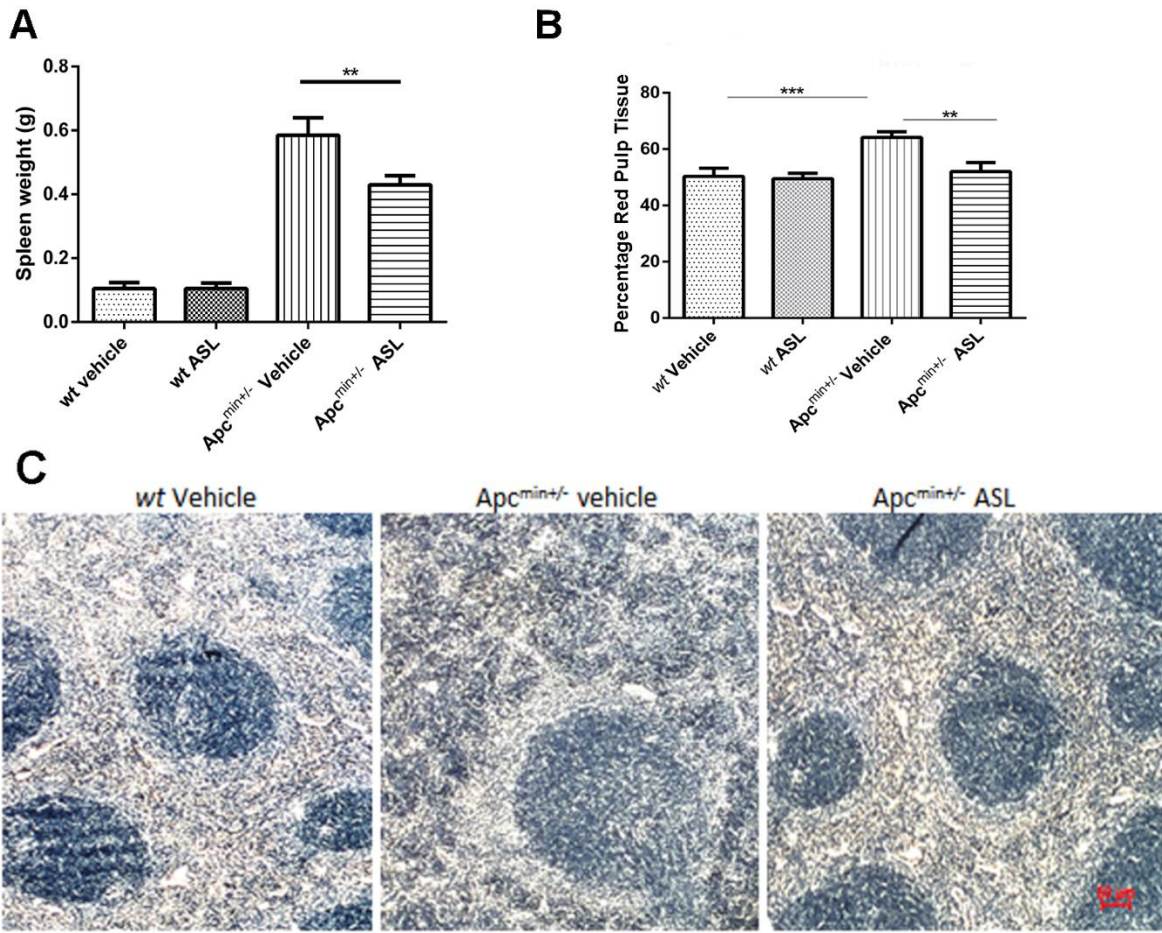
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## Cell Death.tif

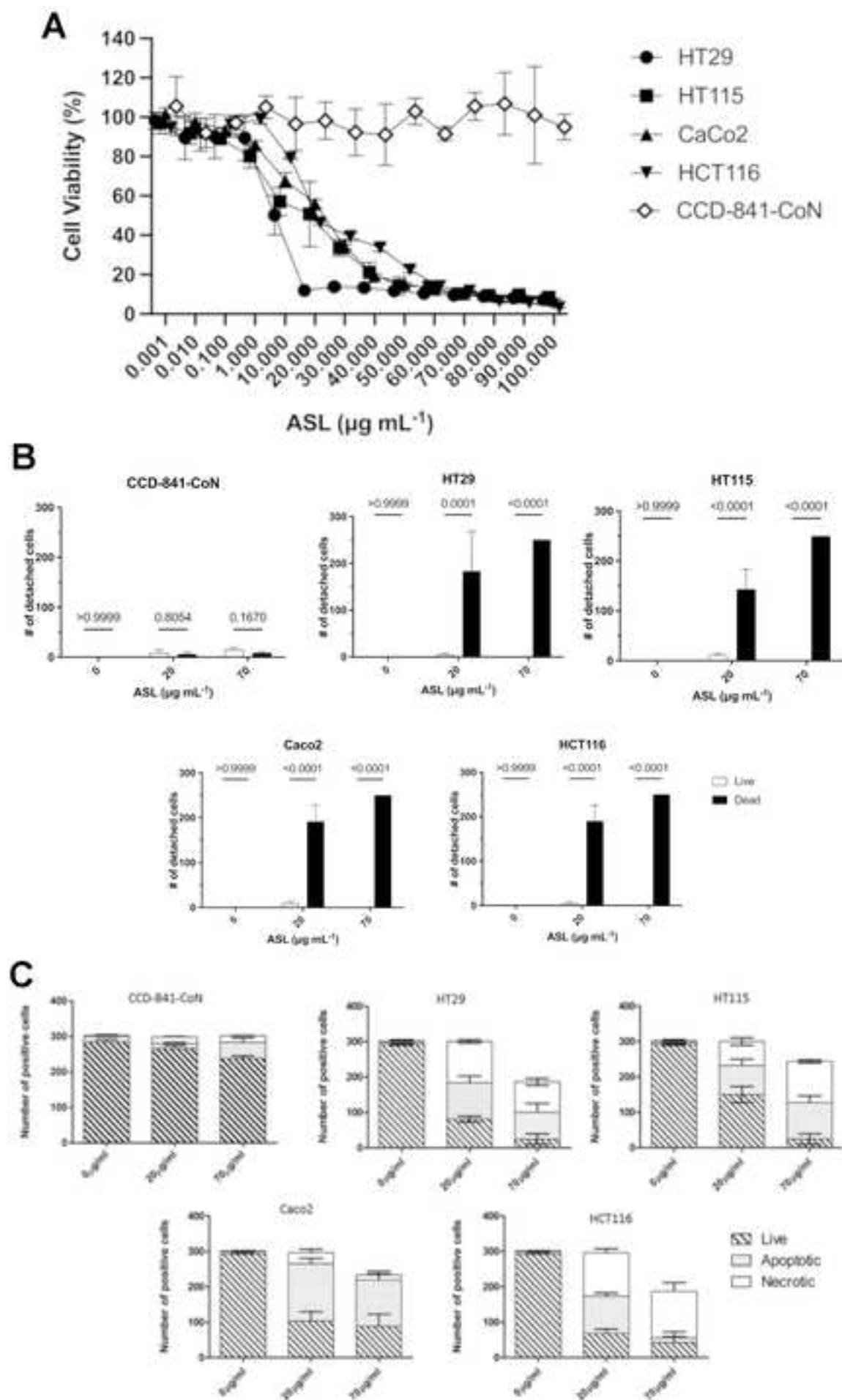




Fig2b\_BoydenChamber

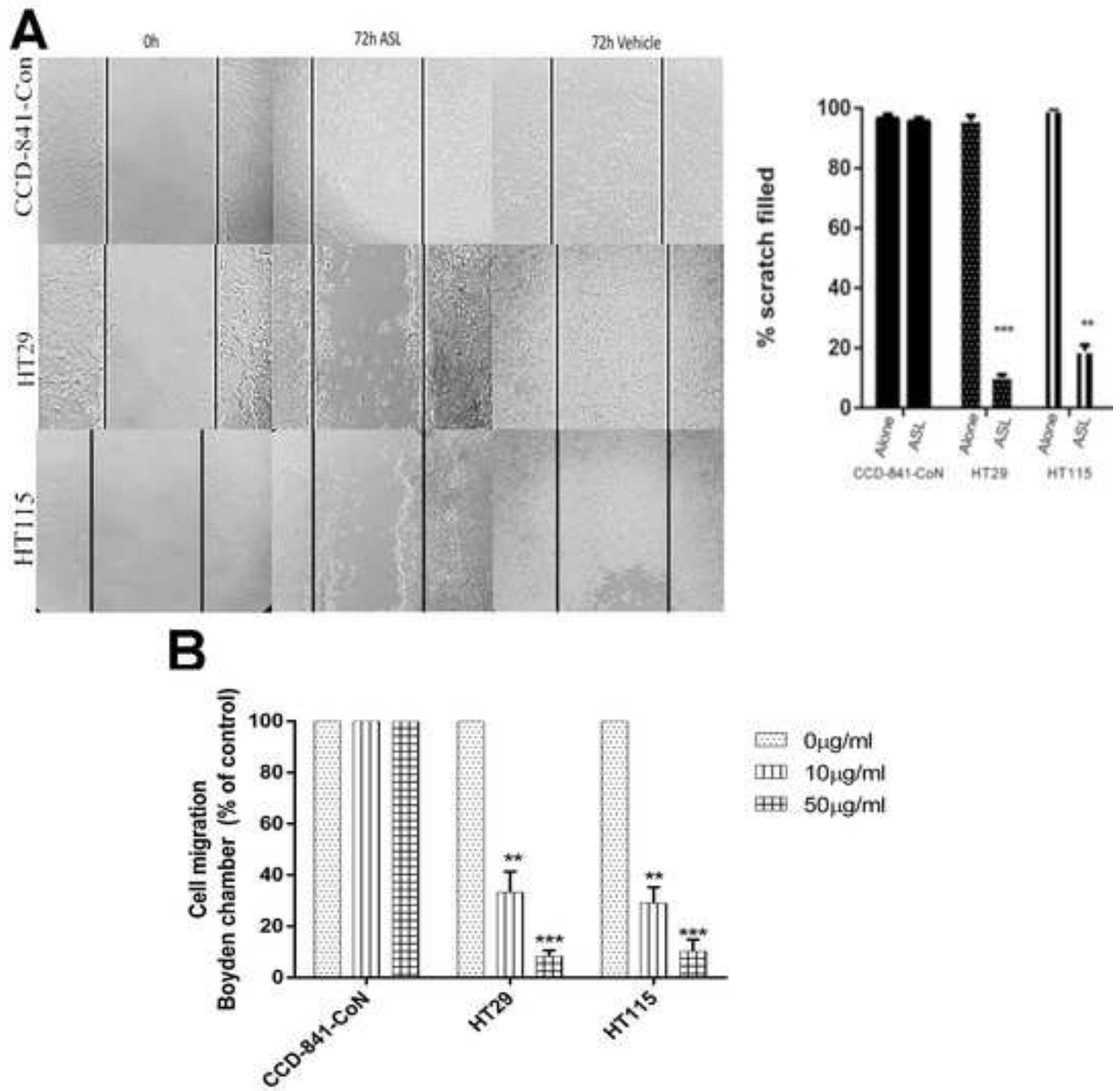
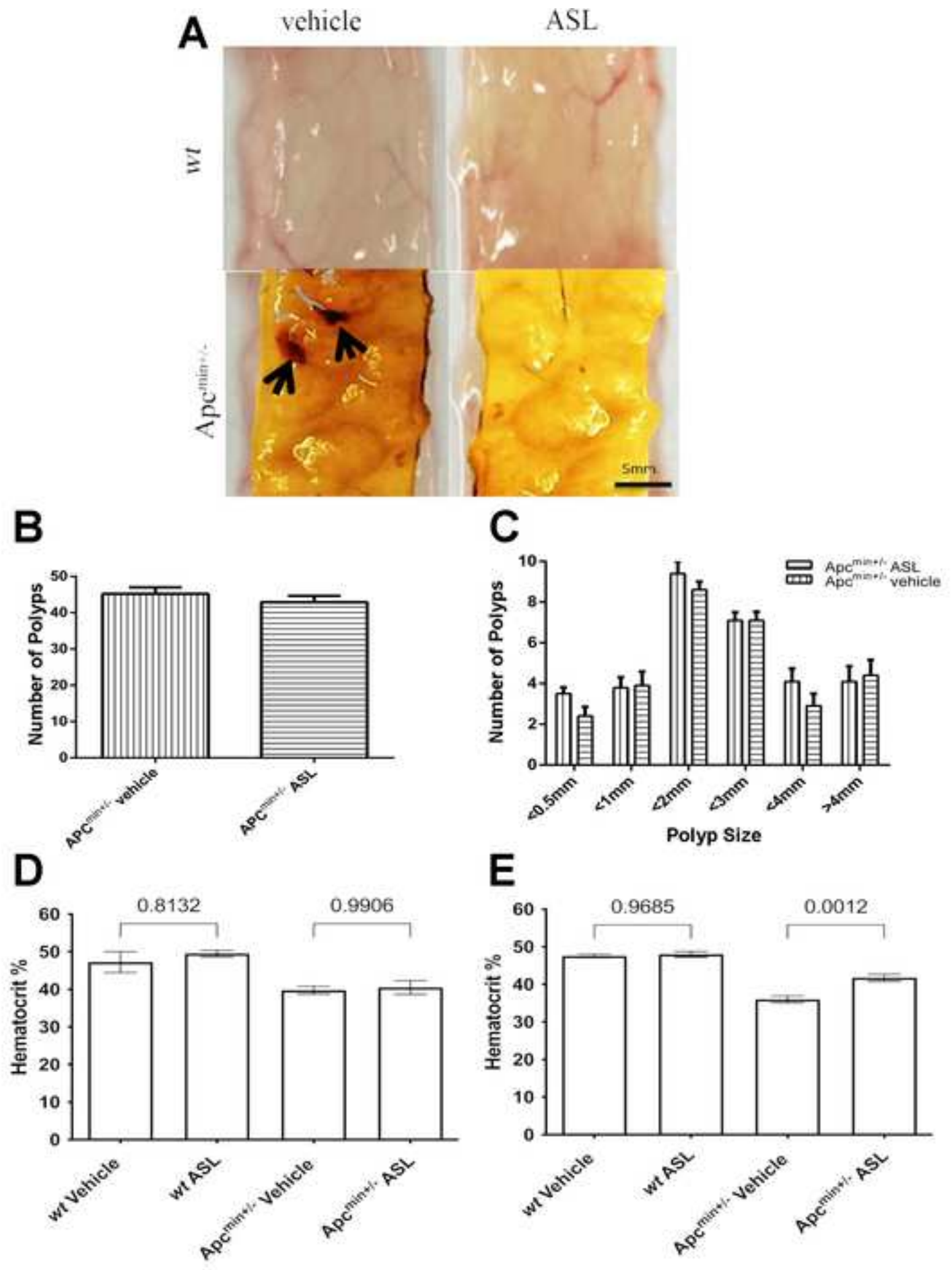
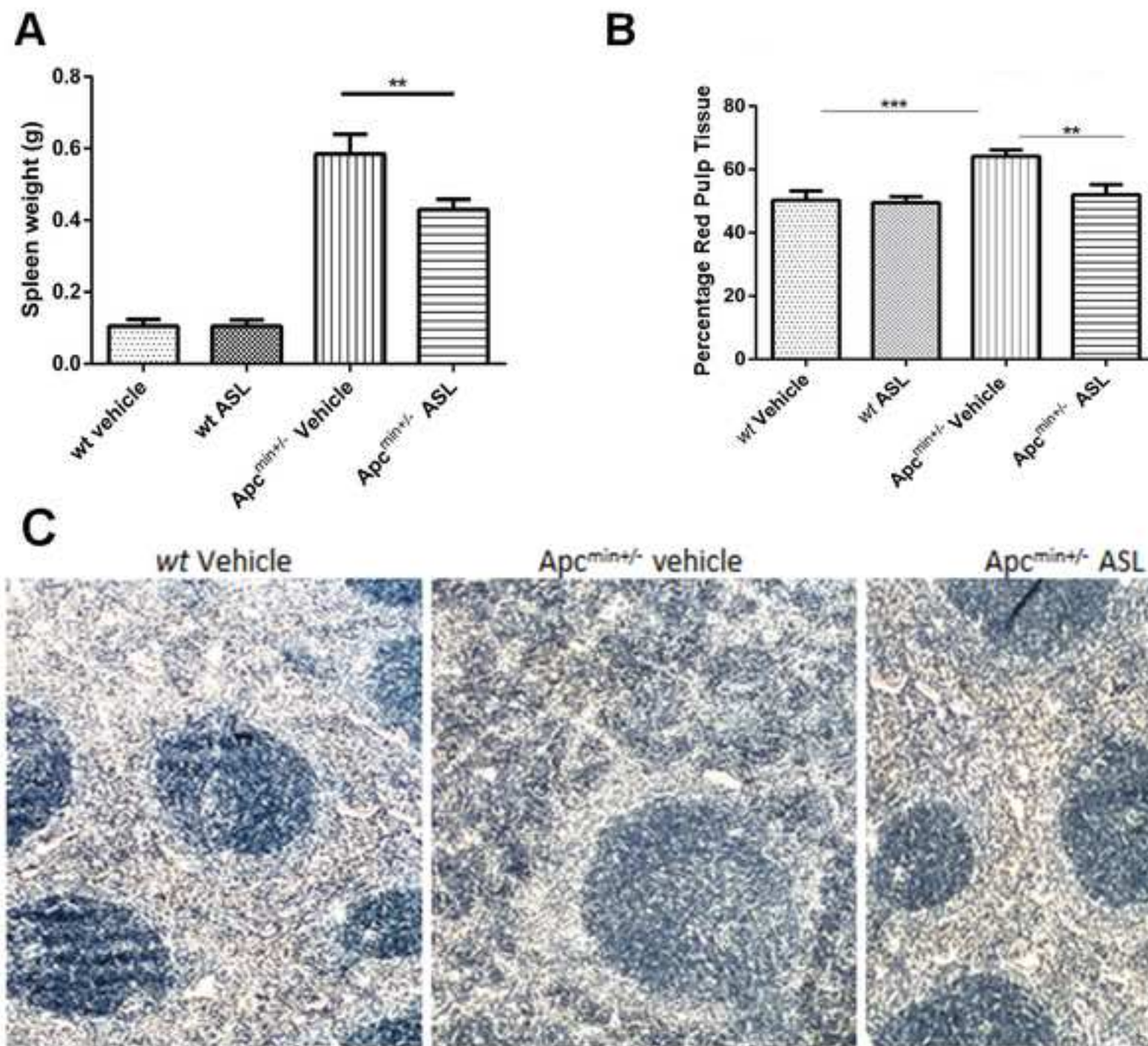
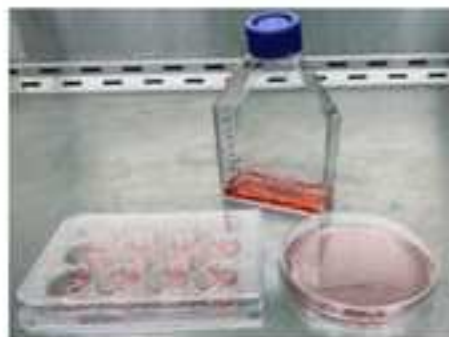


Fig3c\_PolypSize





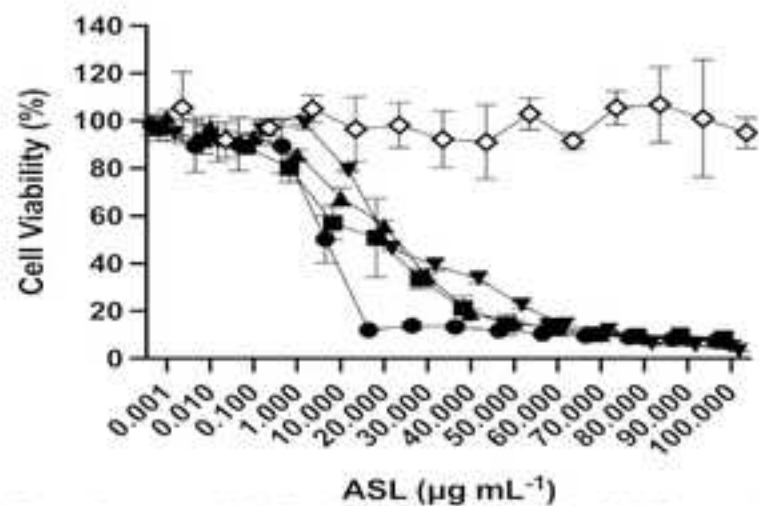
Acidic Sophorolipid



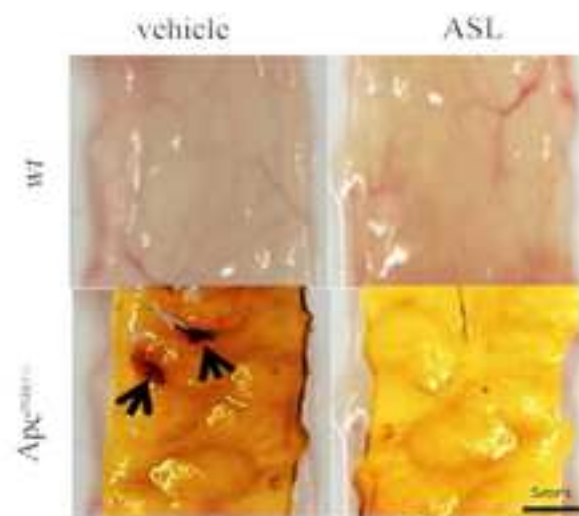
GI Cancer Cell Lines



APC<sup>min+/-</sup> Mouse



Reduced Viability & Cell Death



Reduced Tumor Bleed



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