

*In vitro approaches to assess the effects of açai (Euterpe oleracea) digestion on polyphenol availability and the subsequent impact on the faecal microbiota*

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2 ***In vitro* approaches to assess the effects of Açaí (*Euterpe oleracea*) digestion on**  
3 **polyphenol availability and the subsequent impact on the faecal microbiota**

4

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26 **Abstract.**

27 A considerable proportion of dietary plant-polyphenols reach the colon intact; determining  
28 the effects of these compounds on colon-health is of interest. We hypothesise that both fibre  
29 and plant polyphenols present in açai (*Euterpe oleracea*) provide prebiotic and anti-genotoxic  
30 benefits in the colon. We investigated this hypothesis using a simulated *in vitro* gastrointestinal  
31 digestion of açai pulp, and a subsequent pH-controlled, anaerobic, batch-culture fermentation  
32 model reflective of the distal region of the human large intestine.

33 Following *in vitro* digestion, 49.8% of the total initial polyphenols were available. In mixed-  
34 culture fermentations with faecal inoculate, the digested açai pulp precipitated reductions in  
35 the numbers of both the *Bacteroides-Prevotella* spp. and the *Clostridium-histolyticum*  
36 groups, and increased the short-chain fatty acids produced compared to the negative control.  
37 The samples retained significant anti-oxidant and anti-genotoxic potential through digestion  
38 and fermentation.

39 Dietary intervention studies are needed to prove that consuming açai is beneficial to gut  
40 health.

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43 **Keywords:** Açai pulp, Simulated *in vitro* digestion, Gut microbiota, DNA genotoxicity,

44 Phenolic compounds.

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## 52 1 Introduction

53 A high intake of fruit and vegetables reduces the risk of age related disease (Arts & Hollman,  
54 2005). Mechanistic studies suggest that these effects may be mediated, in part, by the  
55 interaction of undigested plant foods, and not limited to the traditional definitions of dietary  
56 fibre, with the gut microbiota (Marchesi et al., 2016). The prebiotic and health promoting  
57 influences of the plant polyphenols in the colon are of current interest (Williamson &  
58 Clifford, 2010).

59 Açai is an anthocyanin rich Amazonian food with putative benefits to health (Heinrich,  
60 Dhanji, & Casselman, 2011). Intact, the polyphenols present in açai and other polyphenol rich  
61 plant foods may protect against oxidative genotoxic damage, however residual anti-genotoxic  
62 effects through the gut will be dependent on how well the phenolics survive the digestion  
63 process. Ileostomy studies show that polyphenols are not efficiently absorbed in the small  
64 intestine and up to 40 % of those consumed may reach the colon where they are subject to  
65 degradation by the gut microbiota (Tzounis et al., 2011). The products of polyphenol  
66 breakdown are low molecular weight phenolics, such as caffeic acid, which may themselves  
67 have beneficial bioactive effects (Williamson & Clifford, 2010). Estimates of total polyphenol  
68 intake in Europe suggest levels of consumption exceed 1g per day; a better understanding of  
69 the effects of polyphenol rich foods in the gut is therefore desirable (Saura-Calixto, Serrano,  
70 & Goñi, 2007).

71 With a high lipid (~49%) content, açai is a relatively energy dense fruit (Heinrich et al.,  
72 2011). The lipids present are oleic acid (~56.2%), palmitic acid (~24.1%) and linoleic acid  
73 (~12.5%) (Del Pozo-Insfran, Brenes, & Talcott, 2004). Compositionally, açai is also rich in  
74 insoluble fibre with a smaller fraction of soluble fibre (Schauss et al., 2006, Yamaguchi et al.,  
75 2015), it contains ~52.2g/100g DW carbohydrates (Del Pozo-Insfran et al., 2004) and an  
76 abundance of polyphenols in the form of flavonoids and anthocyanins (Gordon et al., 2012). It  
77 is assumed that the lipids and non-fibre carbohydrates are absorbed in the small intestine and

78 that the fibre and a significant fraction of the phenolics survive digestion and may serve as  
79 microbial substrates in the colon.

80 This study aimed to elucidate the probable effects of digested açai on the colonic microbiota  
81 using a model of the digestive process, involving a simulated oral, gastric and then small  
82 intestinal digestion, the removal of small molecules via dialysis, and finally a simulated  
83 colonic fermentation in a pH-controlled, stirred, batch-culture system, with human faecal  
84 inocula, reflecting of the environmental conditions of the distal region of the human large  
85 intestine (Guergoletto, Costabile, Flores, Garcia, & Gibson, 2016). Our primary outcome  
86 measures are induced changes in bacterial groups at the genus level, as measured by  
87 fluorescent *in situ* hybridisation, and changes in short chain fatty acid concentrations. As a  
88 secondary outcome measure, the fates of the polyphenols present in the açai, through each  
89 stage of the process have been followed, along with the anti-genotoxic activity of the  
90 fermentation supernatants produced against a cultured colonic cell line.

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## 104 2 Materials and Methods

### 105 2.1 Chemicals.

106 Agarose, EDTA, trizma base, triton, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), HEPES, and ethidium  
107 bromide were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Sodium chloride (NaCl)  
108 and potassium chloride (KCl) were supplied from Fisher Scientific (Loughborough, UK).  
109 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum  
110 (FBS), 2mM L-glutamine, 100 IU/ml penicillin/streptomycin were obtained from Lonza,  
111 (UK). Phenolic acids standards were obtained from Sigma-Aldrich (UK) and anthocyanin  
112 standards were purchased from Extrasynthese (Genay, France) for HPLC analysis. Other  
113 chemicals and reagents used in this study were obtained from either Sigma Aldrich  
114 (Poole, Dorset, UK) or Fisher Scientific (Loughborough, UK).

### 115 2.2 Açai fruit pulp.

116 Pure açai pulp was harvested from fully ripe fruits in 2013 and was kindly supplied by  
117 Sublime foods (UK). Samples were prepared as described previously (Mills et al., 2008).  
118 In brief, açai pulp (100 g) was freeze-dried upon receipt. The sample was frozen overnight  
119 at -80°C and then dried in an IEC Lyoprep-3000 freeze dryer (Dunstable, UK) for one  
120 week before the samples were ground to powder. Freeze-dried açai was stored at -20°C  
121 until use.

### 122 2.3 Simulated *in vitro* intestinal digestion.

123 A simulated digestion of freeze-dried açai was conducted to look at the potential  
124 bioavailability and bioactivity of açai polyphenols. The digestion followed the protocol  
125 described by Maccaferri et al. (Maccaferri et al., 2012). The remaining digesta was  
126 transferred into a sealed 1 KDa cellulose dialysis membrane (Cheshire biotech Cheshire,  
127 UK,) and dialysed against NaCl (0.01 M, 4 °C) to remove low molecular mass digestion  
128 products and monosaccharides. After 15 hours, the dialysis fluid was changed and dialysis  
129 then continued for a further 2 h. 1 ml aliquots were sampled from the fluids either side of

130 the dialysis membrane, representing the small molecules likely absorbed in the small  
131 intestine and the digesta retained which might then pass to the colon. The retained digesta  
132 was freeze dried (~5days) (Christ- Gamma 2-16 LSC-Germany) in preparation for a  
133 simulated *in vitro* fermentation.

#### 134 **2.4 *In Vitro* Batch Culture Fermentation**

135 The anaerobic batch culture system has been described previously (Ramnani et al., 2012).  
136 Briefly, vessels (working volume 300 mL) were filled with 135 mL of sterile basal nutrient  
137 medium [peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K<sub>2</sub>HPO<sub>4</sub> (0.04 g/L),  
138 KH<sub>2</sub>PO<sub>4</sub> (0.04 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.01 g/L), CaCl<sub>2</sub>·6H<sub>2</sub>O (0.01 g/L), NaHCO<sub>3</sub> (2 g/L),  
139 Tween 80 (2 ml/L), Haemin (0.05 g/L), vitamin K<sub>1</sub> (10 ml/L), L-cysteine (0.5 g/L), bile  
140 salts (0.5 g/L), resazurin (1 mg/L) and distilled water (Sigma Aldrich, UK)]. These vessels  
141 were gassed overnight with O<sub>2</sub>-free N<sub>2</sub> at a rate of 15 ml/min. The temperature of the basal  
142 nutrient medium was set to 37 °C by use of a circulating water bath and the pH was  
143 maintained at 6.8 using a pH controller and NaOH 1M and acidic HCl 1M as necessary  
144 (Electrolab, UK). In order to mimic conditions located in the distal region of the human large  
145 intestine the experiment was run under anaerobic conditions, 37 °C and pH 6.8– 7.0 for a  
146 period of 24 h.

147 Faecal samples were collected from three separate individuals and used as inoculant for the  
148 simulated colonic fermentation of the digested açai. All donors were in good health and had  
149 not taken any antibiotics for at least 6 months before the study; they had no history of bowel or  
150 gastrointestinal diseases. Samples were donated on the morning of the fermentation. The  
151 volunteers were asked to provide these in an anaerobic jar (Anaerojar™ 2.5L, Oxoid Ltd)  
152 which included a gas-generating kit in order to maintain anaerobic conditions. Samples were  
153 diluted (1:10 W/V) with phosphate buffered saline (0.1 M; pH 7.2) and homogenised in a  
154 stomacher (Seward, Norfolk, UK) for 2 min at normal speed. Faecal slurries (15 ml) for each  
155 individual were introduced to three different batch-culture vessels. 1 gram of the freeze dried



156 simulated digesta from the process described above, or a preparation of digesta not  
157 containing açai (as a negative control), was used as a substrate in the batch culture vessels.  
158 Fermentation was conducted for 24 h, and samples were collected at three time points (0, 8,  
159 and 24 hours) for analysis (Figure 22). Samples were stored at -20 °C until analysis.

## 160 **2.5 Identification and quantification of gastric and colonic metabolites.**

### 161 **2.5.1 High-performance liquid chromatography (HPLC).**

162 Phenolic acids and other compounds present in fresh açai pulp, and the phenolics present in  
163 samples following the simulated digestion and following the batch culture simulated colonic  
164 fermentation were assessed via HPLC. Methanol extracts were prepared with samples at each  
165 stage of the digestion model. The extracts were centrifuged at 13,000 g for 10 min and the  
166 filtered through 0.45 µm-acrodisc filters to remove particulates. HPLC was performed using  
167 an Agilent 1100 series HPLC (Hewlett –Packard, Agilent, Bracknell, UK). A Nova Pak C18  
168 column (250 mm × 4.6 mm ID, 5 µm particle size) (Waters Ltd, Elstree, UK) was used to  
169 separate the phenolic constituents. The solvent flow rate was 0.4 ml/min and the column was  
170 allowed to equilibrate for 15 min between each injection. Mobile phase A consisted of 95%  
171 HPLC water, 5% methanol and 0.1% HCl (5 M). Mobile phase B was 50% HPLC water, 50%  
172 acetonitrile and 0.1% HCl (5M). Phenolic compounds were characterised by their retention  
173 time and comparison with known phenolic standards (Rodriguez-Mateos, Cifuentes-Gomez,  
174 Tabatabaee, Lecras, & Spencer, 2012). Detection wavelengths were 280, 254, 320 and 520  
175 nm and all data was analysed using ChemStation software. A standard curve was used to  
176 quantify the amount of each compound.

### 177 **2.6 Total phenolic content.**

178 The total phenolic contents were analysed according to the Folin–Ciocalteu method  
179 adapted to 96-well plate microlitre assay, using gallic acid as the standard; 5 µl of the  
180 diluted extracts or standards were mixed with 145 µl of distilled water and 25 µl of Folin–  
181 Ciocalteu reagent (Sun, Chu, Wu, & Liu, 2002). After 3 min at room temperature, 100 µl

182 of saturated sodium carbonate solution was added and the solution kept in a shaker for 25  
183 min at room temperature. The absorbance of the samples were measured at 650 nm, using  
184 a GENios pro microplater reader (Tecan, Theale, Berks, UK) equipped with the Magellan  
185 Software system. Methanolic solutions of gallic acid (Sigma–Aldrich, Poole, Dorset, UK)  
186 with concentrations of 0–1000 mg/l were used for the calibration curve, and results were  
187 expressed as gallic acid equivalents (GAE) per mg/g of freeze-dried açai starting material  
188 (mean  $\pm$  SD; n = 3, triplicate analysis).

### 189 **2.7 Antioxidant activity using the FRAP assay.**

190 The antioxidant activity of the açai extracts, and of extracts from digested açai, were  
191 determined using the FRAP assay, which is based on the ferric ion reducing power where  
192 the formation of a deep blue complex is readily discernible ( $\text{Fe}^{2+}$ /TPTZ) (Benzie &  
193 Strain, 1996). Serial dilutions of ascorbic acid were prepared as a standard curve (0–  
194 1000  $\mu\text{mol}$ ). 10  $\mu\text{L}$  of solvent extract of samples, or standard, were added to wells of a  
195 96-well plate, followed by 300  $\mu\text{L}$  of FRAP reagent (2.5 mL of 10 mmol/L TPTZ in 40  
196 mmol/L HCl solution and 2.5 ml of 20 mmol/L ferric chloride hexahydrate solution in 25  
197 mL of 0.3 M acetate buffer (pH 3.6)). After a 30 minute incubation, absorbance was  
198 measured at 600 nm using a GENios reader at room temperature with Magellan<sup>TM</sup>  
199 software. The antioxidant capacity was calculated relative to ascorbic acid standards and  
200 expressed as  $\mu\text{M}$  ascorbic acid equivalent/L.

### 201 **2.8 *In vitro* bacterial enumeration by fluorescence *in situ* hybridisation (FISH).**

202 FISH was performed as described by Daims et al. (Daims, Stoecker, & Wagner, 2005).  
203 Briefly, fermentation samples were taken from batch culture vessels at time points: 0, 8, and  
204 24 hours of incubation with digested açai. Oligonucleotide probes, designed to target  
205 specific regions of 16S rRNA, were commercially synthesized and labelled with the  
206 fluorescent dye, Cy3 (Sigma–Aldrich, UK). Bacterial groups enumerated were: Bif164 for  
207 *Bifidobacterium* spp. (Langendijk et al., 1995), Lab158 for *Lactobacillus/Enterococcus*

208 spp. (Harmsen, Elfferich, Schut, & Welling, 1999), Bac303 for *Bacteroides* spp. (Manz,  
209 Amann, Ludwig, Vancanneyt, & Schleifer, 1996), Erec482 for *Clostridium coccoides-*  
210 *Eubacterium rectale* group (Franks et al., 1998), Chis150 for *Clostridium histolyticum*  
211 group and EUB338 for total bacteria (Daims, Brühl, Amann, Schleifer, &  
212 Wagner, 1999).

## 213 **2.9 Short chain fatty acid analysis (SCFAs)**

214 Aliquots of batch culture samples were centrifuged at 13000g for 10 min, and the supernatant  
215 was then filtered through 0.45 µm-acrodisc filters to remove bacteria. 50 µL of supernatant  
216 was injected into the HPLC, with separation of SCFA achieved using an Aminex Ion  
217 Exclusion HPX-87H column (300 mm x 7.8 mm). (Mobile phase, 0.005 M H<sub>2</sub>SO<sub>4</sub>, flow rate  
218 0.6 ml/min, 65°C) with detection of SCFA at a wavelength of 215 nm. Calibration curves  
219 were prepared for acetic, propionic, N-butyric, Iso-butyric and formic acids, with  
220 concentrations between 1.25 mM and 30 mM.

## 221 **2.10 Cell culture**

222 The human HT29 colorectal adenocarcinoma cell line was used as a model for the intestinal  
223 tract. Cells were obtained from the European collection of cell cultures (ECACC) (Salisbury,  
224 UK) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 %  
225 heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml  
226 penicillin/streptomycin. Phosphate Buffered Saline (PBS) and Trypsin- Versene (EDTA)  
227 were purchased from Lonza Ltd (Switzerland). Cells were passaged (21 to 35) twice a week in  
228 a T75 cm<sup>3</sup> cell culture flask (Fisher scientific,UK) and the media was changed every 2 days.  
229 The cells were grown for 5-6 days (approximately 75% confluence) at 37 °C with 5 % CO<sub>2</sub>  
230 and 95% humidity. (Fisher Scientific,UK) (Coates et al., 2007).

## 231 **2.11 Cytotoxicity assay.**

232 HT29 cells were seeded in a 96-well micro-plate Thermo Fisher Scientific Inc, Roskilde,

Denmark) at a concentration of  $1.6 \times 10^5$  (250 $\mu$ l) cells/ml and incubated for 24 hours at 37 °C prior to the assay. Dilutions of filter sterilised fermented açai supernatants (0h and 24h) were prepared in carrier control (at 0%, 1%, 2.5%, 5%, 10% (v/v)) and incubated with cells for 24 hours at 37°C. The medium was removed and 100  $\mu$ L of 4', 6-Diamidino-2 phenylindole dihydrochloride (DAPI) (3mM) was added for 4h at 37°C. The DAPI was removed and absorbance measured at 540 nm using a GENios Pro<sup>TM</sup> micro-plate reader with Magellan<sup>TM</sup>. The experiment was repeated in triplicate in independent experiments. The results were expressed as mean % cell survival normalised to control (without treatment).

## **2.12 Anti-genotoxicity assay, single cell Gel Electrophoresis (Comet assay).**

The assay was carried out following a method described previously (Brown et al., 2012). HT29 cells were adjusted to  $3.2 \times 10^6$  cells ml<sup>-1</sup> (450 $\mu$ l). The cells were incubated with the filter sterilised batch culture fermentation supernatants of the simulated digestion of açai , or with the açai free fermentation as a control (2.5%,250 $\mu$ l) for 24 hours at 37°C. After 24 hours cells were trypsinised and resuspended in media where they were challenged with 75mM H<sub>2</sub>O<sub>2</sub> (100 $\mu$ l) (Sigma-Aldrich, UK) on ice (4°C) for 5 minutes. Cell viability was assessed before and after incubation using Trypan blue. The cells were then centrifuged for 5 minutes at 250 g at 4°C, and the supernatant discarded. The cells were re-suspended in 85 $\mu$ l of 0.85% lower melting agarose (Sigma-Aldrich, UK) in PBS and maintained in a water bath at 43°C. The suspension was mixed well and coated onto frosted slides (pre coated with a layer of 1% normal melting point agarose) (Sigma- Aldrich, UK). The slides were incubated at 4 °C for 10 minutes before being placed in cell lysis buffer (1% Triton X-100 2.5 NaCl, 0.1M EDTA, 0.01M Tris at pH10) for 1 hour at 4°C. The slides were washed with 100 ml of enzyme buffer pH 8.0 (400 mM Hepes, 1M KCL, 5mM EDTA, 2mg/ml BSA Sigma-Aldrich, UK) in a staining jar three times for 5 minutes at 4°C. For base specific assessment of oxidised pyrimidines or purines we applied a (100  $\mu$ l) treatment of either Endonuclease III

259 (10,000 units/ml) (EndoIII) or Formamidopyrimidine-DNA glycosylase (8,000 units/ml)  
260 (FPG) respectively to a subset of the slides post-treatment; control slides were exposed to  
261 enzyme reaction buffer, in the absence of enzymes. The enzyme treated slides were incubated  
262 at 37°C for 45 min with the EndoIII or 30 min with the FPG treatment. The DNA was  
263 allowed to unwind in electrophoresis buffer (10M NaOH, 0.2M EDTA, pH 13.5, Sigma-  
264 Aldrich, UK) at 4°C for 20 minutes before electrophoresis (20 minutes at 26V, 300 MA  
265 (0.037 V/cm). After that, the slides were washed 3 times with neutralising buffer (0.4M Tris,  
266 pH 7.5 with HCl, Sigma-Aldrich, UK) at 4°C for 5 minutes. All slides were stained with 45µl  
267 of ethidium bromide (20µg/ml in PBS) and stored in a dark, moist box at 4°C for no longer  
268 than 48h. Comets were visualised at 400 X magnification using an epifluorescence microscope  
269 (Olympus, Bx51). Fifty cells per slide were analysed and the % tail moments quantified using  
270 Komet 5.5 image analysis software (Kinetic Imaging Ltd, Liverpool, UK). The mean was  
271 calculated for 50 cells from each slide (with each sample in triplicate) and the data were  
272 presented as mean % tail DNA compared to controls (McCann et al., 2007).

### 273 **2.13 Statistical analysis**

274 All statistical analysis was completed using the software PASW18 (SPSS). The microbial  
275 counts and profiles of the faecal samples from the three donors were different at baseline, as  
276 a result mean changes from baseline in the bacterial populations, following fermentation are  
277 reported. Comparisons of each microbial phyla response to treatment or control after  
278 fermentation were made using independent samples T-tests. A one-way analysis of variance  
279 (ANOVA) with a Tukey post-hoc test was conducted to evaluate the changes in microbial  
280 populations with time (0, 8 and 24 hours). The short chain fatty acid data were analysed  
281 using the same approach. An independent samples t-test was used to evaluate comet assay  
282 data. (\*P<0.05, \*\*P<0.01, \*\*\* P<0.001).

283

284

## 285 3 Results

### 286 3.1 Changes in bacterial populations with *in vitro* batch culture fermentation.

287 Fluorescence *in situ* hybridisation was used to assess changes in microbial populations  
288 following batch fermentation of colonic samples at 0, 8 and 24h of fermentation. The  
289 three faecal donors exhibited marked differences in microbial composition at time point  
290 zero, and data are therefore expressed in terms of changes from baseline of specific  
291 phylum following fermentation. The specific effects of fermentation broth enriched with  
292 the digesta from the açai are compared with açai digesta free fermentations. No significant  
293 differences were observed in total bacteria in the fermentations containing the açai digesta  
294 compared to control (Eub338). The inclusion of digested açai pulp inhibited the growth of  
295 *Clostridium histolyticum* group (Chis150) at 8h (with a log  $-0.19 \pm 0.10$  reduction in  
296 bacterial counts from baseline with digested açai, not observed in the control ( $P < 0.05$ ))  
297 and at 24h (with a log  $-0.24 \pm 0.07$  reduction in bacterial counts from baseline with  
298 digested açai, also not observed in the control ( $P < 0.05$ )) (Figure 1). *Bacteroides-*  
299 *Prevotella* spp. (Bac303) counts were also reduced following the inclusion of digested  
300 açai pulp in the fermentation broth at both 8 h (reduction in counts from baseline by log -  
301  $0.14 \pm 0.11$ , not observed in the control ( $P < 0.05$ )) and at 24 h (with a reduction in cell  
302 counts from baseline of log  $-0.09 \pm 0.07$ ) not observed for the negative control ( $P < 0.05$ ))  
303 Figure 1. At the phylum level, no significant differences were observed for the  
304 *Bifidobacterium* spp., the *Lactobacillus/Enterococcus* spp., or the *Clostridium-coccoides*  
305 *Eubacterium rectale* group

### 306 3.2 Short-chain fatty acid production.

307 The short-chain fatty acid (SCFA) concentrations of the supernatants produced during  
308 fermentation were analysed by High Performance Liquid Chromatography (HPLC).  
309 SCFA concentrations in the batch culture at 8h and 24h of fermentation for açai pulp and  
310 negative control are shown in Figure 2. Inclusion of digested açai pulp in the

311 fermentation broth resulted in increased concentrations of total SCFA and of acetic acid at  
312 8 and 24 hours ( $P<0.001$ ), propionic acid at 24 hours ( $P<0.01$ ) and butyric acid at 24  
313 hours ( $P<0.05$ ) relative to control Figure 2.

### 314 **3.3 Degradation of polyphenols during digestion and fermentation by human faecal** 315 **bacteria and their antioxidant capacity.**

316 Major phenolic compounds present in açai were identified by HPLC, samples were taken  
317 through the simulated digestion model to follow the degradation of these compounds prior to  
318 batch culture fermentation (Table 1). The main phenolic compounds present in açai pulp  
319 prior to the digestion were p-hydroxybenzoic acid followed by gentisic acid, chlorogenic acid,  
320 caffeic acid, syringic acid, ferulic acid, trans-cinnamic acid, quercetin and vanillic acid,  
321 cyanidin-3-O-glucoside cyanidin-3-O-rutinoside, pelargonidin-3-O-glucoside and peonidin-  
322 3-O-rutinoside. All of these were still present following the simulated gastric digestion.  
323 However, after the intestinal digestion, considerable degradation of specific polyphenols had  
324 occurred with the anthocyanin concentration decreasing markedly compared to the original  
325 açai pulp. After dialysis peonidin-3-O-rutinoside was not detected. Following the dialysis  
326 process gentisic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, trans-  
327 cinnamic acid and quercetin were recovered albeit in concentrations markedly reduced from  
328 those observed with the raw açai. As measured by the folin ciocalteau assay the availability  
329 of total phenolics had reduced by 51% following the digestion of the açai pulp (Table 1).  
330 Following dialysis, the digested açai was added to fermentation media and fermented in  
331 batch cultures using human faecal samples as inocula, samples of the fermentation  
332 supernatant were collected at 0, 8 and 24h analysed using HPLC for individual phenolics  
333 (Table 2) and for total phenolics via Folin Ciocalteau. After 24 hours of fermentation, 7  
334 polyphenols were recovered, p-hydroxybenzoic acid, chlorogenic acid, ferulic acid, quercetin  
335 acid, and vanillin acid. Their concentrations decreased progressively with incubation time. No  
336 phenolic compounds were detected in the control samples (standard fermentation broth).

337 Undigested açai, with its very high anthocyanin content had considerable antioxidant  
338 capacity as observed in the FRAP assay. With the simulated digestion and destruction of  
339 polyphenols the antioxidant capacity fell by 57% and following fermentation the total  
340 antioxidant capacity was 34% of that of the undigested açai. Batch culture fermentation  
341 supernatants with the digested açai did however have a higher total antioxidant capacity than  
342 the control fermentation supernatant (Table 2). Sugar and dietary fibre were analysed by  
343 Campden BRI laboratories (AOAC Method AC-203). The açai pulp total fibre content was  
344 15.5 g/100 g (15.0g/100 insoluble) and 1.6 g /100 g of sugar. After the digestion the açai  
345 pulp had a remaining total fibre content of 10.2 g/ 100g and a sugar content of 1.0g /100g.

#### 346 **3.4 Cytotoxicity of fermented açai digests.**

347 HT29 cells were treated with filter sterilised fermentation supernatants at a concentration of  
348 0%, 1%, 2.5%, 5% and 10% (v/v) in carrier control media for 24 hours. There were no  
349 cytotoxic effects after 24 hours for either the açai or the carrier control fermentations at  
350 concentrations of 1% and 2.5% v/v. However, at 5% and 10% concentrations, viability fell  
351 to 80% and to 56% respectively. The 2.5% v/v dilution was therefore considered to be most  
352 appropriate for further investigation (Supplemental Figure 1).

#### 353 **3.5 Anti-genotoxicity (Comet assay)**

354 The comet assay (Single Cell Gel Electrophoresis) was used to assess DNA damage. Filter  
355 sterilised samples were prepared from the fermentation broths, collected at 0 hours of  
356 fermentation, or following 24 hours of fermentation. These supernatants were applied at  
357 2.5% v/v in carrier control to HT-29 cells and incubated for 24 hours, DNA damage was then  
358 induced by subsequent exposure to 75  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 5 minutes. A significant anti-genotoxic  
359 effect ( $P < 0.05$ ) was observed for the fermentation supernatant containing the digested açai at  
360 0 hours, with a reduction in DNA damage of approximately 31.5% ( $6.66 \pm 1.2$  %) verses the  
361 açai free fermentation supernatant used as a negative control ( $9.73 \pm 0.8$  % tail DNA) (Figure  
362 3). Moreover, after 24h incubation the fermentation supernatant from the açai digesta



363 conferred protection against oxidised purines in the FPG modified assay ( $10.27 \pm 0.5$  % vs 13  
364  $\pm 0.2$  % tail DNA) ( $P < 0.05$ ) Figure 3.

#### 365 4 Discussion.

366 Here, we present data modelling the effects of consuming of açai on the gastrointestinal  
367 tract. In the first instance, our work demonstrates that polyphenols present in açai may be  
368 degraded during the digestion process, but importantly, that they are not fully destroyed and  
369 a significant percentage of these compounds may therefore reach the colon. In our simulated  
370 colonic environment, these phenolics were shown to be further degraded, and alongside the  
371 dietary fibre present in açai, they may influence the composition of the gut microbiota, with  
372 resultant increases in the synthesis of SCFA. Finally, the presence of digested açai in the  
373 fermentative model of the colon is shown to confer protection against genotoxic insult in  
374 what is an otherwise carcinogenic environment.

375 Food ingredients escaping digestion in the upper GI tract, which then go on to selectively  
376 stimulate the growth and activities of beneficial gut microflora, such as *bifidobacteria* and  
377 *lactobacilli*, over less desirable groups, such as *Clostridium histolyticum*, may be considered  
378 prebiotic (Gibson, 1998). We used a 24h pH-controlled batch-culture fermentation model,  
379 inoculated with human faecal microbiota, to examine the prebiotic potential of digested açai  
380 pulp. We found an inhibition in the growth of *Bacteroides-Prevotella* and *Clostridium*  
381 *histolyticum* when digested açai was incorporated into culture media. *Bacteroides* are a  
382 dominant species in the gut, and whilst largely symbiotic and sacchorolytic, they can be  
383 opportunistic pathogens (Sánchez-Patán et al., 2012). In contrast, within the *Clostridium*  
384 *histolyticum* group there are some well-studied pathogens, such as *Clostridium perfringens*,  
385 as such, a reduction in this microbial group is considered to be a positive modulation of the  
386 microbiota. The reduction in the observed relative counts of *Bacteroides-Prevotella* and  
387 *Clostridium histolyticum* with the digested açai may result from the availability of substrate  
388 which is preferentially utilised by other bacterial groups, alternatively polyphenols in the  
389 culture media may be selectively inhibiting microbial growth (Tzounis et al., 2008). Phenolic  
390 compounds and their metabolites have previously been shown to inhibit the growth of

391 harmful bacteria (Cueva et al., 2013), although further work is need to understand our  
392 observation.

393 The production of SCFA, as a result of saccharolytic fermentation, in the large intestine is  
394 also of benefit to the health of the host (Collins & Gibson, 1999). SCFAs serve as substrates  
395 for energy metabolism in the colonic epithelia (Gibson, 1998), they may also reduce the  
396 growth of pathogens, exert anti-cancer and anti-inflammatory activities, and they serve as  
397 signalling molecules in the gut-brain axis, influencing metabolism and satiety (Gibson,  
398 Probert, Van Loo, Rastall, & Roberfroid, 2004). In this study, the total SCFA, and the acetic  
399 acid, propionate and butyrate concentrations were all higher in supernatants from the  
400 fermentations with the digested açai pulp. Acetic acid is the main product of saccharolytic  
401 fermentation by bifidobacteria and bacteroides (Collins & Gibson, 1999). As our bacteriodes  
402 count decreased with the digested açai, and there were no significant differences between the  
403 numbers of bifidobacteria in treatment versus control, the observed higher concentrations of  
404 acetate may reflect the utilization of lactic acid by other microorganisms, thus producing  
405 acetate via cross feeding (Hernandez-Hernandez, Côté, Kolida, Rastall, & Sanz, 2011).

406 Polyphenols present in açai are credited with the high anti-oxidant capacity of this food  
407 (Pacheco-palencia, Hawken, & Talcott, 2007). We recently completed an acute human  
408 dietary intervention study with acai, in which we observed reduced total plasma oxidant  
409 capacity following acai consumption (Alqurashi et al., 2016). This is consistent with the  
410 recent findings of Pala et al. also suggesting that acai phenolics or their metabolites are  
411 bioavailable *in vivo* (Pala et al., 2017). Henning et al. have shown that açai retains its anti-  
412 oxidant capacity post *in vitro* digestion (Henning et al., 2014). However, to our knowledge,  
413 ours is the first study to investigate the fate of açai polyphenols during the processes of both  
414 digestion and fermentation in a model of the intestinal tract. Notably, we report that the anti-  
415 oxidant capacity may be retained in the colon following interaction with the microbiome.  
416 This observation was of particular interest; in our previous human dietary intervention study,  
417 we observed spikes in improvements in arterial function at 2 hours and again at 6 hours post

418 ingestion of acai, strongly suggesting the liberation of bioactives from ingested acai in the  
419 colon (Alqurashi et al., 2016). The quenching of oxidative stress in the gut prevents DNA  
420 damage in colonic epithelia, and thus may protect against cancer at that site, and our previous  
421 human intervention trial suggests benefits to vascular health from acai as a consequence of  
422 gut microbial function.

423 During the intestinal digestion ~50% of the total phenolic compounds were destroyed with  
424 considerable biotransformation and degradation of individual phenolics. The resistance of  
425 phenolics to digestion is understood to be related to structural sensitivity to enzymes and  
426 acid. We observed survival of some of the anthocyanins as far as the batch culture  
427 fermentation phase of the model; however no anthocyanins were recovered post  
428 fermentation. Anthocyanins are hydrolysed by the intestinal microbiota to small phenolic  
429 acids which may be absorbed in to the blood stream where they seemingly influence health  
430 (Keppler & Humpf, 2005) (Vitaglione et al., 2007).

431 Having identified an increased antioxidant capacity, and higher levels of phenolics, and of  
432 SCFA in the gut model fermentation supernatants, we were interested in potential anticancer  
433 activity. The COMET assay is a widely used, semi-quantitative measure of DNA damage  
434 which is considered an early event in the cancer process. In the colonic HT29 cell line,  
435 exposure to our filter-sterilised batch-culture fermentation products inhibited peroxide  
436 induced DNA damage. The colonic environment in man is carcinogenic (Sánchez-Patán et  
437 al., 2012), dietary exposures which attenuate latent genotoxicity in the colon are therefore  
438 desirable. Dietary intervention studies with both polyphenol, and with dietary fibre, rich  
439 foods lead to lower faecal sample genotoxicity as assessed against HT29 cells in the comet  
440 assay (Eid et al., 2015). A similar dietary intervention study with açai is needed to confirm  
441 that this may be of benefit to the consumer.

442 Our study utilizes an *in vitro* model to mimic gastrointestinal transformations which are  
443 otherwise challenging to capture *in vivo*. These models have been widely used and are well  
444 published. The batch culture fermentation system has been developed to screen the effects of

445 foods and nutrients on human colon microbiota (Salminen et al., 1998). However, as a model  
446 it simulates only the conditions of the distal colon, with pH control mimicking conditions at  
447 that site but limiting the success of species which thrive at lower pH (Faber, Fahey Jr,  
448 Paeschke, & Aimutis, 2011). Furthermore, there is no clearance of substrate/and or  
449 fermentation products via the absorptive process, as would occur *in vivo*; the build-up of  
450 product in the fermentation vessel affects microbial activity and metabolite production.  
451 However this work provides insight into mechanisms through which açai consumption might  
452 influence both digestive and systemic health, and provides evidence to justify further  
453 intervention.

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**633 7 Figure Captions.****634 Figure 1.**

635 Change from baseline in bacterial phyla as analysed by fluorescence in situ hybridisation  
636 (FISH) in a batch culture fermentation containing digested açai fruit or a negative control at 8  
637 and 24 h of fermentation. Results are reported as the mean of three independent fermentations  
638 with faecal samples from three different donors used as inoculate (n = 3) in log 10 CFU/ml ±  
639 standard deviations (SD). Significant differences between treatment and control fermentation  
640 are reported using a t-test \*P<0.05, \*\*P<0.01).

641 **Figure 2.** Mean change in concentration from baseline for the Short chain fatty acids  
642 produced during the fermentation of pre-digested açai fruit or a negative control in a pH-  
643 controlled batch culture with faecal inoculate. Results are reported as the mean of three  
644 independent fermentations with faecal samples from three different donors used as inoculate  
645 (n = 3). Significant differences between treatment and control fermentation are reported  
646 using a t-test (using t-test, \*\*\*P<0.001, \*\*P<0.01 \*P<0.05).

647 **Figure 3.** The anti-genotoxic effects of fermentation supernatants containing digested açai or  
648 a negative control (acai free digesta fermentation) on HT-29 cells (COMET assay). HT-29  
649 cells were incubated with filter sterilized mixed-culture fermentation supernatants (sampled  
650 at 0h & 24h of the fermentation) for 24h before challenge with H<sub>2</sub>O<sub>2</sub> (75µM for 5 min). Data  
651 representing mean % tail DNA, from three experimental runs involving different faecal  
652 inocula donors. The buffer only treatment represents global DNA damage, The EndoIII  
653 enzyme was used to assess oxidised pyrimidines. The FPG enzyme was used to assess  
654 oxidised purines. (\*= p<0.05 independent sample T-test).

655 **Supplemental Figure 1.** Cytotoxicity of fermentation supernatants containing açai  
656 presented as mean ± SD percent cell viability of HT-29 cells (n=3). HT-29 cells  
657 were incubated with filter sterilised supernatant sampled at 0h (A) and at 24 hours  
658 (B) of the fermentation. The percentage of cells surviving the treatment was  
659 determined by DAPI staining.

