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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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Abstract.

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

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

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

Keywords: Açai pulp, Simulated *in vitro* digestion, Gut microbiota, DNA genotoxicity, Phenolic compounds.

1 Introduction

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

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

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

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

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

2 Materials and Methods

2.1 Chemicals.

Agarose, EDTA, trizma base, triton, hydrogen peroxide (H_2O_2), HEPES, and ethidium bromide were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Sodium chloride (NaCl) and potassium chloride (KCl) were supplied from Fisher Scientific (Loughborough, UK). Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 IU/ml penicillin/streptomycin were obtained from Lonza, (UK). Phenolic acids standards were obtained from Sigma-Aldrich (UK) and anthocyanin standards were purchased from Extrasynthese (Genay, France) for HPLC analysis. Other chemicals and reagents used in this study were obtained from either Sigma Aldrich (Poole, Dorset, UK) or Fisher Scientific (Loughborough, UK).

2.2 Açai fruit pulp.

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

2.3 Simulated *in vitro* intestinal digestion.

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

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

2.4 *In Vitro* Batch Culture Fermentation

The anaerobic batch culture system has been described previously (Ramnani et al., 2012). Briefly, vessels (working volume 300 mL) were filled with 135 mL of sterile basal nutrient medium [peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $MgSO_4.7H_2O$ (0.01 g/L), $CaCl_2.6H_2O$ (0.01 g/L), $NaHCO_3$ (2 g/L), Tween 80 (2 ml/L), Haemin (0.05 g/L), vitamin K_1 (10 ml/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L) and distilled water (Sigma Aldrich, UK)]. These vessels were gassed overnight with O_2-free N_2 at a rate of 15 ml/min. The temperature of the basal nutrient medium was set to 37 °C by use of a circulating water bath and the pH was maintained at 6.8 using a pH controller and NaOH 1M and acidic HCl 1M as necessary (Electrolab, UK). In order to mimic conditions located in the distal region of the human large intestine the experiment was run under anaerobic conditions, 37 °C and pH 6.8− 7.0 for a period of 24 h.

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

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

2.5 Identification and quantification of gastric and colonic metabolites.

2.5.1 High-performance liquid chromatography (HPLC).

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

2.6 Total phenolic content.

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

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

2.7 Antioxidant activity using the FRAP assay.

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

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

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

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

2.9 Short chain fatty acid analysis (SCFAs)

Aliquots of batch culture samples were centrifuged at 13000g for 10 min, and the supernatant was then filtered through 0.45 µm-acrodisc filters to remove bacteria. 50 µL of supernatant was injected into the HPLC, with separation of SCFA achieved using an Aminex Ion Exclusion HPX-87H column (300 mm x 7.8 mm). (Mobile phase, 0.005 M H_2SO_4, flow rate 0.6 ml/min, 65°C) with detection of SCFA at a wavelength of 215 nm. Calibration curves were prepared for acetic, propionic, N-butyric, Iso-butyric and formic acids, with concentrations between 1.25 mM and 30 mM.

2.10 Cell culture

The human HT29 colorectal adenocarcinoma cell line was used as a model for the intestinal tract. Cells were obtained from the European collection of cell cultures (ECACC) (Salisbury, UK) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM *L*-glutamine,100 IU/ml penicillin/streptomycin. Phosphate Buffered Saline (PBS) and Trypsin- Versene (EDTA) were purchased from Lonza Ltd (Switzerland). Cells were passaged (21 to 35) twice a week in a T75 cm^3 cell culture flask (Fisher scientific,UK) and the media was changed every 2 days. The cells were grown for 5-6 days (approximately 75% confluence) at 37 °C with 5 % CO_2 and 95% humidity. (Fisher Scientific,UK) (Coates et al., 2007).

2.11 Cytotoxicity assay.

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

Denmark) at a concentration of 1.6 x 10^5 (250µl) 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 µL 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^{TM} micro-plate reader with Magellan^{TM}. 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 3.2x10^6 cells ml^{-1} (450µl). 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%,250µl) for 24 hours at 37˚C. After 24 hours cells were trypsinised and resuspended in media where they were challenged with 75mM H2O2 (100µl) (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 85µl 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 ^{o}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 µl) treatment of either Endonuclease III

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

2.13 Statistical analysis

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

3 Results

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

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

3.2 Short-chain fatty acid production.

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

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

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

Major phenolic compounds present in açai were identified by HPLC, samples were taken through the simulated digestion model to follow the degradation of these compounds prior to batch culture fermentation (Table 1). The main phenolic compounds present in açai pulp prior to the digestion were p-hidroxybenzoic acid followed by gentisic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, trans-cinnamic acid, quercetin and vanillic acid, cyanidin-3-O-glucoside cyanidin-3-O-rutinoside, pelargonidin-3-O-glucoside and peonidin-3-O-rutinoside. All of these were still present following the simulated gastric digestion. However, after the intestinal digestion, considerable degradation of specific polyphenols had occurred with the anthocyanin concentration decreasing markedly compared to the original açai pulp. After dialysis peonidin-3-O-rutinoside was not detected. Following the dialysis process gentisic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, trans-cinnamic acid and quercetin were recovered albeit in concentrations markedly reduced from those observed with the raw açai. As measured by the folin ciocalteau assay the availability of total phenolics had reduced by 51% following the digestion of the açai pulp (Table 1).

Following dialysis, the digested açai was added to fermentation media and fermented in batch cultures using human faecal samples as innocula, samples of the fermentation supernatant were collected at 0, 8 and 24h analysed using HPLC for individual phenolics (Table 2) and for total phenolics via Folin Ciocalteau. After 24 hours of fermentation, 7 polyphenols were recovered, p-hidroxybenzoic acid, chlorogenic acid, ferulic acid, quercetin acid, and vanillin acid. Their concentrations decreased progressively with incubation time. No phenolic compounds were detected in the control samples (standard fermentation broth).

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

3.4 Cytotoxicity of fermented açai digests.

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

3.5 Anti-genotoxicity (Comet assay)

The comet assay (Single Cell Gel Electrophoresis) was used to assess DNA damage. Filter sterilised samples were prepared from the fermentation broths, collected at 0 hours of fermentation, or following 24 hours of fermentation. These supernatants were applied at 2.5% v/v in carrier control to HT-29 cells and incubated for 24 hours, DNA damage was then induced by subsequent exposure to 75 μM H2O2 for 5 minutes. A significant anti-genotoxic effect ($P<0.05$) was observed for the fermentation supernatant containing the digested açai at 0 hours, with a reduction in DNA damage of approximately 31.5% (6.66 ± 1.2 %) verses the açai free fermentation supernatant used as a negative control (9.73 ± 0.8 % tail DNA) (Figure 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 ± 0.5 % vs 13

364 ± 0.2 % tail DNA) ($P<0.05$) Figure 3.

4 Discussion.

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

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

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

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

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

Our study utilizes an *in vitro* model to mimic gastrointestinal transformations which are otherwise challenging to capture *in vivo*. These models have been widely used and are well 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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7 Figure Captions.

Figure 1.

Change from baseline in bacterial phyla as analysed by fluorescence in situ hybridisation (FISH) in a batch culture fermentation containing digested açai fruit or a negative control at 8 and 24 h of fermentation. Results are reported as the mean of three independent fermentations with faecal samples from three different donors used as inoculate (n = 3) in log 10 CFU/ml ± standard deviations (SD). Significant differences between treatment and control fermentation are reported using a t-test *P<0·05, **P<0·01).

Figure 2. Mean change in concentration from baseline for the Short chain fatty acids produced during the fermentation of pre-digested açai fruit or a negative control in a pH-controlled batch culture with faecal inoculate. Results are reported as the mean of three independent fermentations with faecal samples from three different donors used as inoculate (n = 3). Significant differences between treatment and control fermentation are reported using a t-test (using t-test, ***P<0.001, ***P*<0·01 **P*<0·05).

Figure 3. The anti-genotoxic effects of fermentation supernatants containing digested açai or a negative control (acai free digesta fermentation) on HT-29 cells (COMET assay). HT-29 cells were incubated with filter sterilized mixed-culture fermentation supernatants (sampled at 0h & 24h of the fermentation) for 24h before challenge with H_2O_2 (75µM for 5 min). Data representing mean % tail DNA, from three experimental runs involving different feacal innocula donors. The buffer only treatment represents global DNA damage, The EndoIII enzyme was used to assess oxidised pyrimidines. The FPG enzyme was used to assess oxidised purines. (*= p<0.05 independent sample T-test).

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

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