**SUPPLEMENTARY MATERIAL**

**In *vitro* colonic fermentation of ultrasonicated blackberry (*Rubus fruticosus*) residues cv. Tupy**

**Plant Foods for Human Nutrition**

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**Material and Methods.**

**Sample.** Blackberries (*Rubus fruticosus*) cv. Tupy fruits were obtained from Atotonilco el Grande, Hidalgo, Mexico. Fruit was used to prepare the juice and the blackberry residue (BR) was obtained. This was lyophilized (7753020, LABCONCO, MO, USA), milled (IKA® A11 basic, Wilmington, USA) and sieved to obtain a particle size of 500 μm. Sample was stored in sealed plastic bags at −30°C until further analysis [1].

**Ultrasound Treatment.** A solution at 4% (w/v) of lyophilized sample with deionized water (16 g/400 mL) was prepared and treated with ultrasound (VCX-1500, USA, 1500 W and 20 kHz) using a probe of 25 mm at conditions of amplitude and time (91% by 15 min, respectively) and 2 s duration pulse on and 4 s off. Later, the sample was placed in a chamber with a jacket vessel with water circulation (≈ 4°C) to control the temperature produced by the treatment of the ultrasound [2]. Then, the sample was centrifuged (Allegra 25™, Beckman Coulter, CA, USA) at 10,000 rpm for 30 min, at 4°C and supernatant (ultrasonicated blackberry residues (US-BR)) was lyophilized and stored at -30°C until further analysis.

***In vitro* Colonic Fermentation.** Before *in vitro* colonic fermentation, *in vitro* bioaccessibility was performed [3] to take the non-bioaccessible fraction. This was lyophilized and used for *in vitro* colonic fermentation methodology, which was developed by means in a batch culture system under strict anaerobic conditions (using carbon dioxide) for 24 h. *In vitro* fermentation method was described by Barry et al. [4] and standardized by Goñi and Martin-Carrón [5], with minor modifications. Male Wistar rats with mean weight of 200 ± 5 g fed with standard maintenance diet were supplied by the Bioterio at Institute of Health Sciences (Autonomous University of Hidalgo State, Mexico). The inoculum was obtained from the cecum of rats, the cecal content was weighed and added to a flask containing sterile anaerobic medium (1:10 w/v). This was mixed and filtered before use. The anaerobic medium contained trypticase, micromineral solution, buffer, macromineral and resazurin (as anaerobic redox indicator) [6]. The anaerobic medium (16 mL) was added to the 50 mL Erlenmeyer flask that contained 100 mg of non-bioaccessible fraction; it was left for 16 hr at 4°C. After, 2 mL of inoculum was added to the Erlenmeyer flask and then incubated in a water bath with shaking (Labtech LSB-015S, Korea) at 37°C for 24 hr and 60 rpm in anaerobic conditions. The pH (210, Hanna Instruments, USA) and lactic acid bacteria (LAB) were measured before and after incubation. Two controls containing lactulose (L-7877 Sigma-Aldrich, USA) and other without substrate, were included as completely fermentable and zero fermentable substrate, respectively. At the end of time, the fermentation was stopped with NaOH (1 M). Samples were centrifuged (Allegra 25, Beckman Coulter, USA) at 10,000 rpm by 10 min at 25°C, the supernatants and residue were collected and stored at -30°C for further analysis. The supernatants were divided for the assays of short chain fatty acids (SCFA) and flavonoids, while the residue was used for determination of non-fermentable residue and disappeared organic matter.

**Non Fermented Residue (NFR).** According to Goñi and Martin-Carrón [5], the non-fermentable residue (NFR) is the fraction that is not used by the microbiota intestinal and that is excreted, this was calculated as follow:

NFR (mg) = Sample residue weight - blank no substrate residue weight

**Determination of Disappeared Organic Matter (DOM).** The disappeared organic matter is the prediction of the production of final metabolites [5] and was calculated according to Goñi et al. [7] as follow and expressed as percentage (%):

DOM (%) = ((mg of substrate – mg NFR)/mg substrate)\*100

where:

substrate = Initial dry substrate weight

NFR = Non fermented dry substrate weight

**pH and Lactic Acid Bacteria (LAB).** The pH was measured using a potentiometer (model pH 210, Hanna instruments, Microprocessor pH-meter, USA). Lactic acid bacteria (LAB) were counted at time 0 and 24 h after *in vitro* colonic fermentation. Serial dilutions were performed in peptone water solution. 100 μL of samples were taken and diluted in 900 μL of peptone water (DIBICO, Mexico) previously sterilized (Wacs-1080, Wise Key, USA). 20 μL of each dilution was taken and the seeding was carried out by extension on a plate on Man, Rogosa and Sharpe (MRS) agar (DIBICO, Mexico) and was incubated at 37ºC for 24 h. The results were expressed as log colony forming units per milliliter (log CFU/mL) [8].

**Short Chain Fatty Acids (SCFA) Analysis.** The method of Goñi and Martín-Carrón [5] for the short chain fatty acids (SCFA) with some modifications was used. 400 μL of aliquot of supernatant from the fermentation samples plus 50 μL of internal standard (2-ethylbutyric acid, Sigma-Aldrich, USA), 50 μL of phosphoric acid (Fisher, Scientific, USA) and 500 μL of Milli-Q water. The mixture was stirred and filtered (0.22 µm, 33 mm, SLGV033RB, Millex-GV, PVDF, Ireland). 1 µL was injected manually (Microliter, 701, HA-80330, Hamilton, USA) on gas chromatograph (Perkin Elmer, Clarus 500, USA) which had a capillary column DB-FFAP (Free Fatty Acid Phase, 15 m x 0.53 mm x 0.50 µm, Agilent J&W GC, USA) and a detector flame ionization [9]. The initial oven temperature was 70°C, maintained for 2 min, raised to 180°C at 16°C/min and held for 1 min, then was increased to 200°C at 20°C/min, and finally held at 200°C for 15 min. Helium as a gas carrier was used. The temperature of the detector and injector was 240°C. SCFA were identified and quantified by comparison with known fatty acid standards (acetic, propionic and butyric acid (Sigma-Aldrich, USA)). SCFA produced were expressed as milligrams per liter (mg/L).

**Flavonoids by High Performance Liquid Chromatography (HPLC) Analysis.** The quantification of flavonoids was carried out following the methodology proposed by Saura-Calixto et al. [10] with slight modifications. The samples fermented by the intestinal microbiota were treated prior to their quantification. Twenty milligrams of lyophilized fermented samples were previously taken and suspended in 500 µL of deionized water and stirred. Subsequently, a solid phase extraction (SPE) was carried out by means of Oasis HLB cartridge (60 mg, 3 cc, WAT094226, Waters, USA) and was activated with 1 mL of methanol (Meyer, Mexico) and 2 mL of deionized water. The sample was then placed inside the cartridge and washed with 9 mL of water containing 5% methanol and 2% glacial acetic acid (Meyer, Mexico), with the objective of removing the interfering components. The compounds of interest (flavonoids) were eluted with 1 mL of methanol. Subsequently, the methanol was evaporated with nitrogen to dryness and reconstituted with 200 µL of aqueous solution of acetic acid (HPLC grade, Sigma-Aldrich, USA) and adjusted to pH 2.8 (initial mobile phase of HPLC). The samples were passed through a 0.45 μm Gelman filter (GHP, hydrophilic polypropylene membrane, 4426, Acrodisc 13 mm, Syringe Filters, Pall Life Sciences, USA) and stored at -20 °C until analysis.

The flavonoids were quantified by HPLC according to the methodology established by Díaz-Batalla et al. [11] with modifications, using a detector with diode array (Waters 2996, USA) with autosampler (Waters 717, USA) and a Symmetry C18 column (5 μm x 4.6 mm x 250 mm, WAT054275, Waters, Ireland). Mobile phase A was acetonitrile (JT Baker, USA) and mobile phase B was deionized water to a pH of 2.8 adjusted with acetic acid grade HPLC (Sigma-Aldrich, USA). The volume of injection was 20 μL and individual phenolic compounds standards (Sigma-Aldrich, USA) were used as reference. The gradient conditions for the elution of flavonoids were as follows: 35% B in 10 min; 20% B in 15 min; 5% B in 16 min; 4% B in 17 min; 80% B in 18 min; 100% B in 20 min. While that the gradient conditions for the elution of phenolic acids were: 50% B in 16 min; 35% B in 18 min; 5% B in 20 min; 4% B in 21 min; 80% B in 22 min and 100% B in 24 min. UV-Vis spectra were used to detect flavonoids and phenolic acids. The absorbance for myricetin, quercetin, apigenin and kaempferol was at 252, 254, 266 and 265 nm, respectively. Phenolic acids as gallic, caffeic, vanillic, coumaric, ferulic, protocatechuic, and cinnamic were measured at 269, 323, 260, 309, 322, 259, and 274 nm, respectively.

**Scanning Electron Microscopy (SEM).** In order to identify morphological changes of BR and US- BR, before and after *in vitro* colonic fermentation were evaluated by scanning electron microscopy. Samples were deposited on the silicon wafer and coated with gold (Denton Vacuum Desk V, USA) applying 20 millitorr and 20 mA during 4 min. Samples were observed in a scanning electronic microscopy (JEOL JSM-6300, USA) at 250x and 500x amplifications [12].

**Statistical Analysis.** The data were obtained by triplicate and expressed as mean ± standard deviation (SD). Data were analyzed performing a one-way analysis of variance (ANOVA) and differences among means were determined using a Tukey test with a level of significance of *p*<0.05. Also, the t-Student test was used at the same significance level. The statistical package SPSS® System for WIN™ version 15.0 was used.

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