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Comparative *in vitro* fermentations of cranberry and grape seed polyphenols with colonic microbiota

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ABSTRACT

In this study, we have assessed the phenolic metabolism of a cranberry extract by microbiota obtained from the ascending colon and descending colon compartments of a dynamic gastrointestinal simulator (SHIME). For comparison, parallel fermentations with a grape seed extract were carried out. Extracts were used directly without previous intestinal digestion. Among the 60 phenolic compounds targeted, our results confirmed the formation of phenylacetic, phenylpropionic and benzoic acids as well as phenols such as catechol and its derivatives from the action of colonic microbiota on cranberry polyphenols. Benzoic acid (38.4 μg/ml), 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (26.2 μg/ml) and phenylacetic acid (19.5 μg/ml) reached the highest concentrations. Under the same conditions, microbial degradation of grape seed polyphenols took place to a lesser extent compared to cranberry polyphenols, which was consistent with the more pronounced antimicrobial effect observed for the grape seed polyphenols, particularly against *Bacteroides*, *Prevotella* and *Blautia coccoides–Eubacterium rectale*.

1. Introduction

The first clinical study into the effect of consumption of cranberry (*Vaccinium macrocarpon* or *Oxycoccus macrocarpus*) in treating urinary tract infections (UTIs) dates back to 1966 (Papas, Brusch, & Ceresia, 1966). Since then, most of these studies reported a preventive effect against UTIs (for review, see Vasileiou, Katsargyris, Theocharis, & Giaginis, 2013), although in some particular studies no significant effects were observed (Barbosa-Cesnik et al., 2011; Stapleton et al., 2012).

The red cranberry is rich in several groups of phenolic compounds, especially flavonols (200–400 mg/kg), anthocyanins (136–1710 mg/kg) and proanthocyanidins (PACs) (4188 mg/kg) (Pappas & Schaich, 2009). PACs are oligomers and polymers of flavan-3-ol monomers [mainly (epi)afzelechin, (epi)catechin and (epi)galocatechin] joined by B-type (4β-8 or 4β-6) and additional A-type (2β-O-7 or 2β-O-5) linkages. Oligomeric forms with at least one A-type interflavanic linkage – which awards certain conformational inflexibility to the molecule – predominate in cranberry PACs (Pappas & Schaich, 2009). Besides polyphenols, other phytochemicals occurring in cranberries are terpenes, organic acids, complex carbohydrates, and sugars (Pappas & Schaich, 2009). The beneficial effects of cranberry against UTIs have been attributed, at least partly, to their PAC content and special composition, although the most active structures has still not been elucidated (Shmuely, Ofek, Weis, Rones, & Houri-Haddad, 2012; Vasileiou et al., 2013). Other foods only containing B-type PACs, such as grape seeds or apples, lack these preventive properties against UTI exhibited by cranberry. Cranberry A-type PACs have been shown to exhibit uropathogenic *Escherichia coli* (UPEC)-anti-adhesive activity and other activities related to bacterial interaction with host cells to a greater extent than B-type PACs (Feliciano, Meudt, Shanmuganayagam, Krueger, & Reed, 2014), but PACs are unlikely to appear in urine at relevant concentrations as they are poorly absorbed in the intestines. One leading hypothesis is that cranberry components, and/or their direct metabolites, present in the urine would operate in the phase of bacterial adherence of UPEC to the uroepithelial cells, preventing bacterial colonization and progression of UTIs (Vasileiou et al., 2013). In fact, *ex vivo* studies confirmed the anti-adhesive activity of urine

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samples collected from volunteers who consumed cranberry products in comparison to urine samples collected from the placebo group (Howell et al., 2010), suggesting that the compounds responsible for the benefits against UTIs might be products of the metabolism of the cranberry (and/or not-metabolized cranberry components) eliminated in the urine.

With the final aim of looking into the metabolism of cranberry polyphenols in more depth and unraveling the potential mechanisms behind the selective and preventive effects of cranberry consumption against UTIs, we have carried out comparative batch culture fermentations of cranberry and grape seed extracts with colonic microbiota. For these fermentations, human microbiota from the colonic compartments of the dynamic simulator of the human intestinal microbial ecosystem (SHIME) (Molly, van de Woestijne, & Verstraete, 1993) was used. Production of phenolic acids and other related metabolites were monitored over 48 h to assess differences in the metabolic profiles of cranberry and grape seed extracts subjected to the same microbiota and fermentation conditions. Microbial community analyses and microbial metabolic activity (short-chain fatty acids and ammonium production) determinations were also conducted to determine the effects of both extracts on gut microbiota survival.

2. Materials and methods

2.1. Phenolic standards and extracts

Standards of phenolic compounds were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) and Extrasynthèse (Genay, France). A commercial cranberry extract was kindly supplied by Triarco Industries Inc. (NJ, USA). Total phenolic content of the cranberry extract was 219 mg of gallic acid equivalents/g, as measured by the Folin–Ciocalteu reagent (Merck, Darmstadt, Germany). The cranberry extract contained benzoic acids (9.76 mg/g), hydroxycinnamic acids (11.1 mg/g), flavan-3-ols (2.1 mg/g) and anthocyanins (0.055 mg/g) (sample #18 in Sánchez-Patán et al., 2012b). A commercial grape seed extract (Vitaflavan®) was kindly provided by Les Dérives Resiniques & Terpéniques (DRT), S.A. (France). Total phenolic content of the grape seed extract was 629 mg of gallic acid equivalents/g, as measured by the Folin–Ciocalteu reagent. The grape seed extract mainly contained flavan-3-ols (337 mg/g), including galloylated and non-galloylated forms (Sánchez-Patán et al., 2012a).

2.2. In vitro batch incubations with human colonic bacteria

In vitro batch incubations were performed by sampling 25 ml of the ascending colon and descending colon compartments (AC and DC, respectively) (~8 log copy number/ml) of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This dynamic *in vitro* gastrointestinal model comprises a series of five double-jacketed fermentation vessels simulating the stomach, small intestine and the three-stage large intestine conditions (Molly et al., 1993). The colon compartments contained *in vitro* cultured microbiota that harbored a reproducible human microbial community representative of the *in vivo* conditions, both in composition and metabolic activity (Van den Abbeele et al., 2010). Following sampling, the colon microbial suspensions (25 ml) were placed into bottles containing cranberry or grape seed extracts (500 mg/l) and were incubated for 48 h at 37 °C. To obtain anaerobic conditions, L-cysteine (0.5 g/l) was added and bottles were closed with butyl rubber stoppers and flushed with N_2 during 15 cycles of 2 min each at 800 mbar over pressure and 900 mbar under pressure. Before starting the incubation, bottles were placed at atmospheric pressure. Samples were taken at 0, 6, 24 and 48 h with a needle that extends beyond the butyl rubber stoppers that seal off the incubation bottles. Upon sampling, the mixture was flushed with N_2 to ensure anaerobic conditions. Samples were centrifuged (10000 rpm, for 10 min at 4 °C) and pellets and supernatants were stored at −20 °C until further analysis. Just before analysis, samples were defrosted and pellets were used for DNA isolation, and supernatants were filtered (0.22 μm) and analysed for phenolic metabolites, short-chain fatty acids and ammonium. For each extract, three independent experiments were carried out.

2.3. Targeted analysis of phenolic acids and other metabolites

Phenolic compounds were analyzed by a previous UPLC–ESI-MS/MS method (Jiménez-Girón et al., 2013). Filtered supernatants were directly injected into the UPLC equipment. The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size from Waters (Milford, MA, USA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient programme was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total run-time of 18 min. The flow rate was set constant at 0.5 ml/min and injection volume was 2 μl. The LC effluent was pumped to a Waters Acquity TQD tandem quadrupole mass spectrometer (Milford, MA, USA) equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 l/h; cone gas (N_2) flow rate, 60 l/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón et al., 2013). All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric, 4-hydroxy-5-(3′-hydroxyphenyl)-valeric and 4-hydroxy-5-(phenyl)-valeric acids, which were quantified using the calibration curves of 3-(3′,4′-dihydroxyphenyl)-propionic, 3-(3′-hydroxyphenyl)-propionic and propionic acids, respectively. Data acquisition and processing were realized with MassLynx 4.1 software. Injections were carried out in duplicate.

2.4. Microbial community analyses

Quantitative PCR (qPCR) on total bacteria and different groups and genera of bacteria (*Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Prevotella*, *Enterobacteriaceae*, *Blautia coccoides–Eubacterium rectale* group, *Clostridium leptum* subgroup and *Ruminococcus*) was performed following the methodology described in Barroso et al. (2013). Briefly, bacterial DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) buffer and phenol–chloroform–isoamyl alcohol and bead-beating. The DNA was precipitated with polyethylene glycol (PEG-6000), washed in ice-cold 70% ethanol and dried in a Speed-Vac, prior to resuspension in distilled water. The DNA concentration and quality of the samples were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).