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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).

Bacterial numbers in the DNA samples were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, CA, USA). Primers, amplification conditions and calculation of copy numbers have been detailed previously (Barroso et al., 2013). DNA from *E. coli* DH5α, *Lactobacillus plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of the groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously (Barroso et al., 2013). Samples were subjected to DNA extraction and analyzed in duplicate.

2.5. Analysis of short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA) and ammonium

The SCFA and BCFA were extracted from the samples with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard and extracts were analyzed as described previously (Alander et al., 1999). Briefly, one microliter of the diethyl ether layer was injected and measured in a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a capillary-free fatty-acid packed column [EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 m × 0.53 mm, film thickness 1.2 μm], a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C respectively. The concentration of SCFA and BCFA was calculated in mg/l. Total SCFA were calculated based on the amounts of acetate, propionate, butyrate, valerate and caproate and BCFA based on the amounts of isobutyrate, isovalerate and isocaproate. Samples were analyzed in duplicate.

Ammonium determination was performed as earlier described by Bremner and Keeney (1965). Briefly, ammonium was released from samples as ammonia by the addition of MgO and distillation into a boric acid-indicator solution using an auto distillation Vapodest 30́ (Gerhardt Analytical Systems, Brackley Northants, UK). Ammonia was determined by titration with standard HCl using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium). Ammonium ion concentration was expressed as mg/l. Samples were analyzed in duplicate.

2.6. Statistical analysis

Mean values and standard deviations were calculated based on the values for the different variables during the incubation period (phenolic metabolites, microbial groups, SCFA, BCFA, acetate, propionate, butyrate, and ammonium). Concerning data of phenolic metabolites, three-way analysis of variance (ANOVA) was used to test the main effects of three factors studied (time, compartment, and phenolic extract added). Concerning data of fatty acids, ammonium, and microbial groups, one-way ANOVA and posterior least significant difference (LSD) test, and the corresponding non-parametric Kruskal–Wallis test were used to detect differences along the incubation time, for each compartment and containing either the cranberry or the grape seed extract. The *t*-test for independent samples, together with the corresponding non-parametric Mann–Whitney test, were used to evaluate differences between the phenolic extracts at a certain time of incubation and in each compartment. The significance level considered was $p = 0.05$. Principal component analysis (PCA), from matrix correlation (where variables were previously standardized using all samples) was used to summarize changes in the concentration of microbial-derived phenolic metabolites. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft, Inc. 1984–2006, www.statsoft.com).

3. Results

3.1. Production of phenolic acids and other metabolites

A total of 27 phenolic compounds were identified and quantified in the supernatants from the incubations of cranberry and grape seed extract with human microbiota obtained from the ascending colon and descending colon compartments (AC and DC, respectively) of the SHIME system (Table 1). They corresponded to phenylacetic, phenylpropionic, benzoic and cinnamic acids and phenols, and other metabolites exclusively derived from the catabolism of flavan-3-ols, such as phenyl-γ-valerolactones and phenylvaleric acid derivatives. Among them, benzoic acid (38.4 μg/ml), 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (26.2 μg/ml, quantified as 4-(3′-hydroxyphenyl)-propionic acid) and phenylacetic acid (19.5 μg/ml) reached the highest concentrations (Table 1). A three-way ANOVA analysis was applied to assess the main factors (time, phenolic extract and microbiota) that had significant effect ($p < 0.05$) on the concentration of each phenolic compound quantified (Table 1, Figs. 1 and 2) (Online Supporting Information, Figs. S1–S4). A first result to be noted is that incubation of both extracts with AC and DC microbiota led to a general and steady release of phenylacetic and phenylpropionic acids up to 48 h (Figs. 1 and 2), the time factor being significant in all cases, except for 4-hydroxyacetic and 3-(3′-hydroxyphenyl)-propionic acids (Table 1). For all these phenolic acids, maximum concentrations were found in incubations with the DC microbiota, either because the initial fecal suspension exhibited higher concentration (e.g. phenylacetic acid), or because they were released to a greater extent in the presence of a phenolic extract [e.g. 3,4-dihydroxyphenylacetic and 3-(3′,4′-dihydroxyphenyl)-propionic acids]. The cranberry extract (500 mg/l) led to a significantly higher production of main metabolites, such as 3,4-dihydroxyphenylacetic, 3-(3′,4′-dihydroxyphenyl)-propionic, 3-(4′-hydroxyphenyl)-propionic and phenylpropionic acids when incubated with both AC and DC microbiota, in comparison to the grape seed extract.

Some benzoic and cinnamic acids were present in the phenolic extract per se, as confirmed from the concentration values at $t = 0$. (Online Supporting Information, Figs. S1 and S2). The grape seed extract contained a relevant quantity of gallic acid (3,4,5-trihydroxybenzoic acid) (Sánchez-Patán et al., 2012a) whereas the cranberry extract was specially rich in benzoic, *p*-coumaric (4-hydroxycinnamic), protocatechuic (3,4-dihydroxybenzoic) and vanillic (4-hydroxy-3-methoxybenzoic) acids (sample #18 in Sánchez-Patán et al., 2012b). Time and phenolic extract factors resulted as significant factors for the concentrations of all these benzoic and cinnamic acids during the incubation of the two extracts with the two microbiota (Table 1). Interestingly, gallic acid (grape seed extract) suffered a sharp decrease during the first 6 h of incubation with the AC microbiota, whereas its degradation by the DC was only noticeable after 24 h of incubation (Online Supporting Information, Fig. S1). The same was observed for protocatechuic acid, although to a lesser extent. Similarly, concentrations of cinnamic acids (cranberry extract) constantly decreased over the first 24 h of incubation in the presence of both AC and DC microbiota (Online Supporting Information, Fig. S2). On the other hand, other compounds, such as 2-hydroxybenzoic acid (salicylic acid) and benzoic acid, increased consistently up to 24 h of incubation with both microbiota, but more so in the case of the cranberry extract (Online Supporting Information, Fig. S1).

Table 1
Range of variation (minimum and maximum values) of the concentration of main phenolic metabolites measured during incubations of the cranberry/grape seeds extract with the microbiota taken from the ascending colon (AC) and descending colon (DC) compartments, and significant main effects of the factors.

			Factors' main effects		
	Minimum (μg/ml)	Maximum (μg/ml)	Time	Phenolic extract	Microbiota
Phenylacetic acids					
3,4-Dihydroxyphenylacetic acid	0.00	4.55	*	*	*
3-Hydroxyphenylacetic acid	0.00	0.19	*	–	*
4-Hydroxyphenylacetic acid	0.55	5.75	–	–	*
Phenylacetic acid	4.80	19.54	*	–	*
Phenylpropionic acids					
3-(3′,4′-Dihydroxyphenyl)-propionic acid	0.00	5.32	*	*	*
3-(3′-Hydroxyphenyl)-propionic acid	0.00	1.88	–	–	–
3-(4′-Hydroxyphenyl)-propionic acid	0.00	11.32	*	*	–
Phenylpropionic acid	0.00	1.81	*	*	*
Benzoic acids					
Gallic acid	0.00	7.42	*	*	*
Protocatechuic acid	0.04	6.74	*	*	*
Syringic acid	0.00	0.14	*	*	–
Vanillic acid	0.03	2.54	*	*	–
4-Hydroxybenzoic acid	0.13	0.46	*	*	*
2-Hydroxybenzoic acid	0.08	0.47	*	*	*
Benzoic acid	0.22	38.39	*	*	*
Cinnamic acids					
Caffeic acid	0.00	0.92	*	*	–
Ferulic acid	0.00	0.82	*	*	–
Trans-p-coumaric acid	0.00	5.18	*	*	–
Trans-cinnamic acid	0.00	0.80	*	*	–
Phenols					
Catechol/pyrocatechol	0.00	6.24	*	*	–
4-Metylcatechol	0.00	0.04	–	–	–
4-Ethylcatechol	0.00	0.06	–	–	–
Other metabolites					
1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	0.00	6.09	*	*	*
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	0.00	10.22	*	*	*
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	0.00	15.66	*	*	*
4-Hydroxy-5-(3′-hydroxyphenyl)-valeric acid	0.00	26.20	–	–	–
4-Hydroxy-5-(phenyl)-valeric acid	0.00	15.00	–	–	–

* Statistical significance of the main effects of the factors ($p < 0.05$).

Another group of metabolites formed during the incubation of the extracts with the colon microbiota included catechol/pyrocatechol (1,2-dihydroxybenzene) and its derivatives 4-metylcatechol and 4-ethylcatechol. For all of them, the amount released was greater in the presence of the cranberry extract and their incubation followed similar trends (Online Supporting Information, Fig. S3), although only catechol showed significant differences by time and phenolic extract (Table 1).

Finally, other metabolites derived from the catabolism of flavan-3-ols such as phenyl-γ-valerolactones and phenylvaleric acid derivatives were only detected in the incubations of the grape seed extract with the colonic microbiota (Online Supporting Information, Figs. S4). In the incubations with the DC microbiota, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, which is considered a first intermediate in the catabolism pathway of flavan-3-ols, became detectable at 6 h. The phenyl-γ-valerolactones and phenylvaleric acids were subsequently formed afterwards, again only in the case of the DC incubations. The three main factors (time, phenolic extract and microbiota) were found significant for most of these compounds (Table 1).

Principal component analysis (PCA) was performed in order to summarize changes in the concentrations of phenolic acids and other metabolites resulting from the batch culture fermentations of the cranberry and grape seed extracts with the colonic microbiota. Two principal components (PC1 and PC2), which explained 52.3% of the total variance of the data, were obtained. To show the changes over time, main values of the scores of the triplicate incubations in the different time periods (0, 6, 24 and 48 h) were plotted in the plane defined by the first two principal components (Fig. 3). PC1 (27.3% of total variance explained) was directly correlated (loadings $\geqslant 0.7$) with 3,4-dihydroxyphenylacetic acid (0.961), 3-(3′,4′-dihydroxyphenyl)-propionic acid (0.941), 3-(4′-hydroxyphenyl)-propionic acid (0.932), phenylpropionic acid (0.917), benzoic acid (0.779) and catechol (0.866), all microbial-derived phenolic metabolites produced during the incubation of phenolic extracts. PC2 (25.0% of total variance explained) was inversely correlated with syringic acid (−0.835), vanillic acid (−0.932), caffeic acid (−0.863), ferulic acid (−0.902), *trans*-*p*-coumaric acid (−0.891), and *trans*-cinnamic acid (−0.919), all initially present in the cranberry extract. Main differences among samples were due to the phenolic extract (cranberry/grape seeds) employed, with low PC2 scores for incubations with the cranberry extract (higher concentrations in benzoic and cinnamic acids). Also, changes in the phenolic content during the incubation time were reflected in both PC1 and PC2, as phenolic metabolites were produced (higher scores for PC1) and initial benzoic and cinnamic acids were degraded (higher scores for PC2). Samples from incubations with AC and DC microbiota were closely located for each phenolic extract and followed a similar trend over time, especially for the cranberry extract. Overall, as time progressed, incubations of the cranberry extract with both AC and DC microbiota led to relatively greater changes in their phenolic profile in comparison to the grape seed extract which suffered phenolic metabolism to a lesser extent.

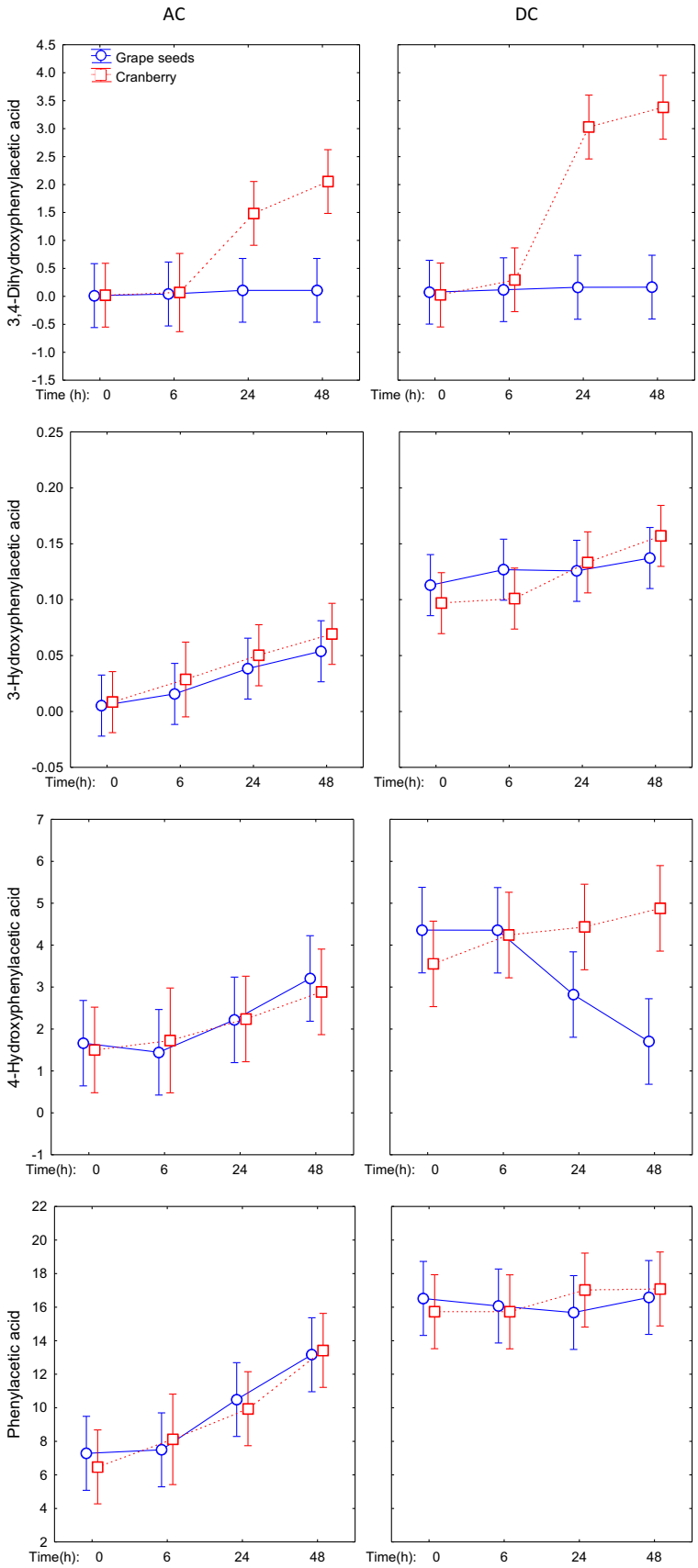

Fig. 1. Mean values of the concentrations of phenylacetic acids (μg/ml) during incubations of phenolic extracts with colonic microbiota: 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and phenylacetic acid. The error bars are 95% confidence intervals ($n = 3$).

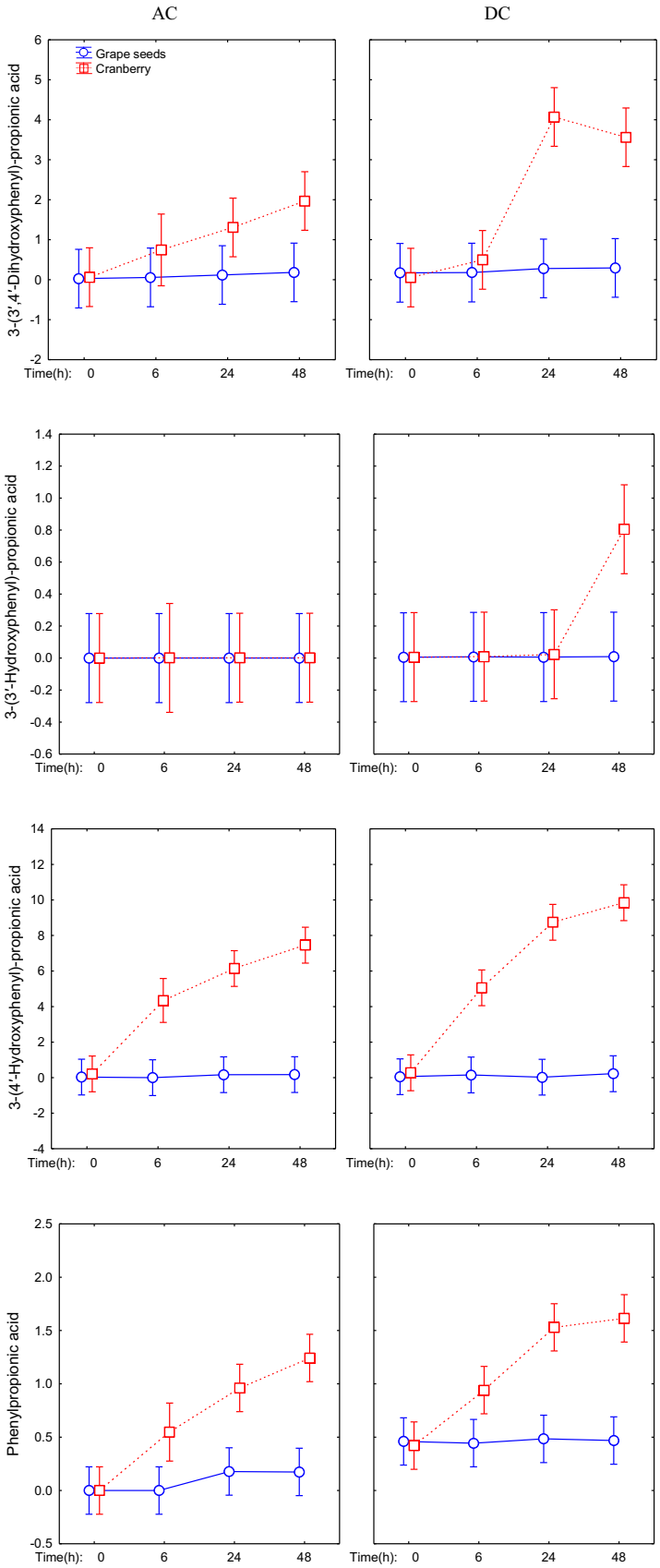

Fig. 2. Mean values of the concentrations of phenylpropionic acids (μg/ml) during incubations of phenolic extracts with colonic microbiota: 3-(3′,4′-dihydroxyphenyl)-propionic acid, 3-(3′-hydroxyphenyl)-propionic acid, 3-(4′-hydroxyphenyl)-propionic acid and phenylpropionic acid. The error bars are 95% confidence intervals ($n = 3$).

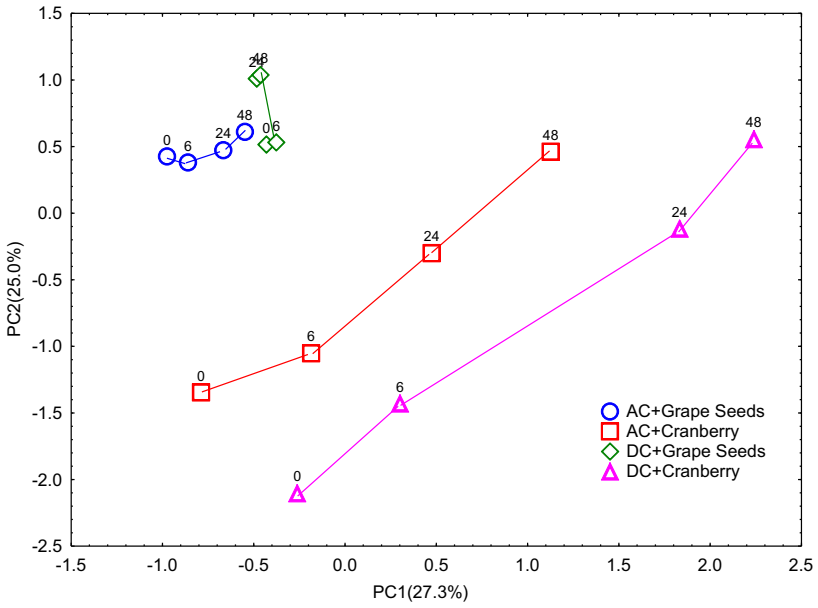

Fig. 3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from a PCA of phenolic compounds from the incubations of phenolic extracts with colonic microbiota at different times (0, 6, 24 and 48 h).

3.2. Changes in microbial counts and metabolism

The impact of the cranberry and grape seed extracts on the AC and DC microbiota from the SHIME was analyzed by qPCR, targeting total bacteria and specific phylogenetic groups (Table 2). Significant differences ($p < 0.05$) in microbiological counts were observed between the AC and DC batches at the incubation onset; *Lactobacillus*, *Bacteroides*, *Prevotella* and *Enterobacteriaceae* being representative of the AC microbiota, whereas *C. leptum* and *Ruminococcus* prevailed in the DC microbiota. Similar results were obtained with the nonparametric Mann–Whitney test. During the batch incubations, counts from all the analyzed bacterial groups generally decreased over time ($p < 0.05$). The reduction of microbial counts could be partially associated with the limitation of substrates during the batch incubations. Moreover, differences in the evolution of microbial groups were observed, depending on the polyphenolic extract analyzed. The decrease of *Enterobacteriaceae* counts was faster when the samples were incubated with cranberry rather than with grape seed, showing a decrease in 1.5 log copy numbers/ml after 24 h of incubation in both the AC and DC batches added with the cranberry extract (Table 2). However, for most of the microbial groups assayed, the highest decrease in counts was observed in the DC batches after 48 h of incubation with the grape seed extract, showing an average decrease in counts of 2 log copy numbers/ml for *Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, *C. leptum* and *Ruminococcus* and of 3 log copy numbers/ml for *Bacteroides*, *Prevotella* and *B. coccoides–E. rectale*. On the other hand, the decrease of the microbial groups in the AC batches was similar during the incubation with both phenolic extracts, showing *Prevotella* as having the highest decrease in counts (about 4 log copy numbers/ml after 48 h of incubation; Table 2). The same significant differences during the batch incubations indicated in Table 2 were obtained using the nonparametric Kruskal–Wallis test, although some for a value of $p < 0.08$.

The effect of the cranberry and grape seed extracts on the fermentative and proteolytic activities of the AC and DC microbial groups was evaluated by the formation of short-chain (SCFA) and branched-chain (BCFA) fatty acids, and ammonium (Table 3). The initial content of SCFA was higher in the samples originated from the DC vessel of the SHIME than those from the AC compartment, whereas the BCFA and ammonium contents showed no significant differences within colonic compartments. Acetic, propionic and butyric acids represented more of the 90% of the SCFA analyzed while isovaleric and isobutyrate acids were the predominant BCFA. During incubation, an increase in the formation of SCFA was only observed in the incubations with the AC microbiota, but no significant differences were observed in the effect of the added polyphenolic extract. The AC batches were characterized as containing the major proportion of saccharolytic bacteria (*Bacteroides*, *Lactobacillus* and *Prevotella*; Table 2), which are mainly responsible of the formation of SCFA. Likewise, the formation of BCFA and ammonium only increased during incubation of the phenolic extracts with the AC microbiota (Table 3). The results obtained with the nonparametric Kruskal–Wallis test matched those of the one-way ANOVA, although four comparisons were statistically significant but for a value of $p < 0.08$.

4. Discussion

Consumption of cranberry is widely recommended as a prophylaxis against UTIs, although the mechanisms behind these preventive effects are still poorly understood. These preventive effects associated with cranberry consumption may be due to the combination of different polyphenols rather than to the action of an individual or single phenolic group alone (Wang, Zuo, Vinson, & Deng, 2012). Therefore, experimentation with cranberry extracts/products rather than with specific cranberry components (i.e. A-type proanthocyanidins), seems an accurate approach to studying their impact on human health. To our knowledge, this is the first *in vitro* study of the degradation of cranberry polyphenols by human microbiota, evaluating both phenolic metabolism by colonic microbiota and the modulation of microbiota by cranberry polyphenols and/or their metabolites. Our results confirmed the formation of phenylacetic (3,4-dihydroxy-, 3-hydroxy-, 4-hydroxy-, and phenylacetic), phenyl-propionic [3-(3′,4′-dihydroxy-phenyl)-, 3-(3′-hydroxy-phenyl)-, 3-(4′-hydroxy-phenyl)- and 3-(phenyl)propionic acid] and benzoic (3,4-dihydroxy-, 4-hydroxy-, 2-hydroxy- and benzoic acid) acids, as well as phenols such as

Table 2
Means ± standard deviation values of the quantitative PCR data (log copy number/ml) for the microbial groups analyzed during incubations of the cranberry/grape seeds extract with the microbiota taken from the ascending colon (AC) and descending colon (DC) compartments.

Bacterial group		Compartment	Incubation time		
			0 h	24 h	48 h
Total bacteria	Cranberry	AC	8.71 ± 0.09c	7.72 ± 0.11b	6.71 ± 0.45a
		DC	8.32 ± 0.33b	7.17 ± 0.40a	6.56 ± 0.50a
	Grape seed	AC	8.61 ± 0.24b	8.38 ± 0.34b	6.64 ± 0.02a
		DC	8.18 ± 0.41b	*7.06 ± 0.51b	5.34 ± 0.80a
Lactobacillus	Cranberry	AC	7.37 ± 0.06c	6.74 ± 0.06b	5.80 ± 0.44a
		DC	*6.78 ± 0.22b	5.68 ± 0.51a	5.54 ± 0.63a
	Grape seed	AC	7.40 ± 0.10c	6.92 ± 0.17b	5.79 ± 0.08a
		DC	*6.80 ± 0.29c	*5.55 ± 0.50b	4.52 ± 0.57a
Bifidobacterium	Cranberry	AC	7.53 ± 0.03b	7.11 ± 0.01b	5.96 ± 0.38a
		DC	6.90 ± 0.45b	6.62 ± 0.65ab	5.83 ± 0.32a
	Grape seed	AC	7.51 ± 0.14b	7.25 ± 0.18b	6.13 ± 0.16a
		DC	6.78 ± 0.59b	5.92 ± 0.82ab	4.84 ± 0.82a
Bacteroides	Cranberry	AC	7.43 ± 0.02c	6.82 ± 0.31b	5.74 ± 0.27a
		DC	*6.87 ± 0.06b	*5.99 ± 0.25ab	5.64 ± 0.89a
	Grape seed	AC	7.51 ± 0.19c	7.22 ± 0.06b	5.62 ± 0.04a
		DC	*7.04 ± 0.15c	*5.81 ± 0.17b	4.01 ± 0.97a
Prevotella	Cranberry	AC	7.74 ± 0.24c	5.38 ± 0.33b	4.15 ± 0.40a
		DC	*6.15 ± 0.20b	*4.44 ± 0.35a	3.98 ± 0.50a
	Grape seed	AC	7.87 ± 0.09c	6.56 ± 0.28b	4.34 ± 0.10a
		DC	*6.41 ± 0.39c	*4.23 ± 0.55b	*3.04 ± 0.57a
Enterobacteriaceae	Cranberry	AC	8.56 ± 0.13c	7.13 ± 0.17b	5.78 ± 0.45a
		DC	*7.52 ± 0.30b	*6.01 ± 0.52a	5.56 ± 0.43a
	Grape seed	AC	8.83 ± 0.03c	8.18 ± 0.11b	6.16 ± 0.26a
		DC	*7.52 ± 0.43c	*6.38 ± 0.47b	5.15 ± 0.72a
B. coccoides–E. rectale	Cranberry	AC	7.34 ± 0.02b	6.97 ± 0.21b	5.82 ± 0.49a
		DC	6.94 ± 0.19c	6.21 ± 0.43b	5.45 ± 0.36a
	Grape seed	AC	7.27 ± 0.03b	7.01 ± 0.13b	5.96 ± 0.19a
		DC	6.95 ± 0.30b	*5.70 ± 0.62b	*4.23 ± 0.85a
Clostridium leptum	Cranberry	AC	5.00 ± 0.06b	5.21 ± 0.44b	4.08 ± 0.61a
		DC	5.98 ± 0.47b	5.11 ± 0.61ab	4.37 ± 0.29a
	Grape seed	AC	5.09 ± 0.25b	5.10 ± 0.26b	3.79 ± 0.30a
		DC	*6.09 ± 0.20b	4.59 ± 0.63a	3.70 ± 0.92a
Ruminococcus	Cranberry	AC	3.98 ± 0.06c	3.57 ± 0.05b	3.01 ± 0.32a
		DC	*5.24 ± 0.19b	4.53 ± 0.42ab	*4.15 ± 0.42a
	Grape seed	AC	3.97 ± 0.04b	3.66 ± 0.04b	2.24 ± 0.68a
		DC	*5.28 ± 0.20c	4.17 ± 0.46b	3.11 ± 0.69a

[a–c]For a given microbial group analyzed, different letters denote significant differences ($p < 0.05$) from LSD test during the incubation time (for a given compartment) with the grape seed and cranberry extracts.
* Denotes differences ($p < 0.05$) between the two compartments for a given incubation time and each extract.

catechol and its derivatives (4-methy- and 4-ethyl) derived from the action of colon microbiota on cranberry polyphenols. Therefore, it would be expected that these metabolites could be absorbed from the large intestine and be further detected in the plasma and urine after the ingestion of cranberry products. Effectively, Wang et al. (2012) found a significant increase in the content of 4-hydroxyphenylacetic, 4-hydroxybenzoic acid, 2-hydroxybenzoic and benzoic acids, among other metabolites, in urine samples of healthy subjects after a 3-week administration of cranberry juice. In the same way, Khanal, Howard, and Prior (2014) found that supplementation of a high-fructose diet with cranberry significantly increased the urinary excretion of 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic, 3-(3′-hydroxyphenyl)-propionic and 3,4-dihydroxybenzoic, among other phenolic acids, in rats.

Another contribution of this study is the comparison between the microbial degradation sequence of cranberry and grape seed polyphenols. Grape seed extracts, rich in B-type proanthocyanidins, have been widely used for the study of the microbial metabolism of flavan-3-ols and their concurrent impact on gut microbiota. In a previous study using the same grape seed extract (Sánchez-Patán et al., 2012a), maximum formation of intermediate metabolites, such as 5-(3′,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid, and of several phenolic acids, including 3-(3,4-dihydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxymandelic acid, and gallic acid was observed at 5–10 h of incubation with fecal microbiota; later phases of the incubations (10–48 h) showed the appearance of mono- and non-hydroxylated forms of previous metabolites by dehydroxylation reactions. Formation of all these metabolites was also detected in the present study, but to a lesser extent, which was attributed to differences in the microbiota and in the incubation conditions used. In any case, in comparison to grape seed polyphenols, microbial degradation of cranberry polyphenols led to a different phenolic metabolic fingerprint, characterized by a relatively higher production of 3,4-dihydroxyphenylacetic, 3-(3′,4′-dihydroxyphenyl)-propionic, 3-(4′-hydroxyphenyl)-propionic and phenylpropionic acids (Table 1, Figs. 1 and 2) (Online Supporting Information, Figs. S1–S4). When all metabolites detected were considered in a statistical multivariable analysis (PCA), we concluded that the extent of microbial degradation was greater in the case of cranberry polyphenols (Fig. 3). These comparative results concerning phenolic metabolism were associated with changes in microbial counts and metabolic activity for both cranberry and grape seed extracts. The lower extent of phenolic metabolism was related with a higher decrease in

Table 3
Mean ± standard deviation data for acetate, propionate, butyrate, total SCFA, BCFA and ammonium during incubation of the ascending colon (AC) and descending colon (DC) compartments with the cranberry and grape seed extracts.

Compound		Compartment	Incubation time		
			0 h	24 h	48 h
Acetate	Cranberry	AC	1877[a] ± 281a	1923 ± 317a	2141 ± 49a
		DC	2497 ± 254a	2586 ± 380a	2370 ± 344a
	Grape seed	AC	1877 ± 282a	1963 ± 213a	2011 ± 70a
		DC	2497 ± 254a	2387 ± 602a	2351 ± 226a
Propionate	Cranberry	AC	761 ± 66a	946 ± 139ab	1014 ± 64b
		DC	993 ± 96a	1006 ± 108a	929 ± 124a
	Grape seed	AC	761 ± 66a	936 ± 124b	975 ± 55b
		DC	993 ± 96a	976 ± 198a	944 ± 39a
Butyrate	Cranberry	AC	399 ± 71a	582 ± 66b	640 ± 58b
		DC	481 ± 12a	486 ± 25a	529 ± 75a
	Grape seed	AC	399 ± 71a	579 ± 57b	604 ± 45b
		DC	481 ± 12a	458 ± 72a	*482 ± 18a
SCFA	Cranberry	AC	3244 ± 453a	3740 ± 568a	4100 ± 156a
		DC	4526 ± 397a	4656 ± 533a	4580 ± 633a
	Grape seed	AC	3244 ± 453a	3762 ± 392a	3905 ± 163a
		DC	4526 ± 397a	4385 ± 946a	4438 ± 390a
BCFA	Cranberry	AC	255 ± 64a	315 ± 35a	329 ± 22a
		DC	356 ± 21a	356 ± 27a	363 ± 47a
	Grape seed	AC	255 ± 64a	319 ± 24a	330 ± 13a
		DC	356 ± 21a	371 ± 54a	367 ± 19a
Ammonium	Cranberry	AC	238 ± 60a	305 ± 19ab	321 ± 30b
		DC	381 ± 8a	348 ± 36a	369 ± 36a
	Grape seed	AC	238 ± 60a	296 ± 6ab	327 ± 11b
		DC	381 ± 8a	*380 ± 24a	358 ± 38a

[a–c]For a given compound analyzed, different lower-case letters, in the same row (compartment), denote significant differences ($p < 0.05$) along the incubation time.
* Denotes differences between the two compartments ($p < 0.05$), for each incubation time, and each extract.
[a] Data at time 0 h correspond to the values analyzed from the AC and DC compartment samples before addition of the phenolic extracts.

microbial counts observed in the DC batches incubated with the grape seed extract (Table 2), and agrees with previous results that demonstrated a certain antimicrobial capacity for this same grape seed extract (Cueva et al., 2013; Tabasco et al., 2011). A comparison of the effect of grape seed and cranberry extracts in the growth of pure cultures of lactic acid bacteria revealed a higher reduction in growth parameters caused by the incubation with grape seed extract (Tabasco et al., 2011) than with cranberry extract (Sánchez-Patán et al., 2012c), showing procyanidin B2 (B-type linkage) to be a higher inhibitory capacity than procyanidin A2 (A-type linkage) (Sánchez-Patán et al., 2012c). Monomeric flavan-3-ols and B-type procyanidin dimers have been described as showing a wide spectrum and higher antimicrobial activity in comparison with other polyphenols (Daglia, 2012).

During the incubation of the colonic microbiota with the phenolic extracts, the groups most affected were *Bacteroides*, *Prevotella* and *B. coccoides–E. rectale*. In a previous study we observed that *Bacteroides* and the *B. coccoides–E. rectale* group were the most inhibited groups after feeding the SHIME colonic microbiota with red wine polyphenols (Barroso et al., 2014). *In vitro* fermentation studies with fecal microbiota have also revealed that (+)-catechin and (epi)gallocatechins were able to inhibit *Clostridium* and *Bacteroides* (Tzounis et al., 2008). On the other hand, the higher counts of microbial groups registered in the AC batches corresponded with a time-dependent increase of the fermentative and proteolytic metabolism observed in these batches (Table 3). *Bacteroides* and *Lactobacillus* are characterized by exhibiting saccharolytic and proteolytic activities (Davila et al., 2013; Ravcheev, Godzik, Osterman, & Rodionov, 2013).

Additionally, this study confirms differences in the metabolic activity of colon microbiota from different regions (i.e., ascending and descending colon). Formation of phenolic acids from the microbial conversion of cranberry and grape seed polyphenols was higher with the DC microbiota, which is in agreement with previous studies (Barroso et al., 2013). Within the microbial groups assayed, the clostridial cluster IV, represented by *C. leptum* and *Ruminococcus*, prevailed in the DC batches. So far, only a few bacterial species, some of them belonging to the class *Clostridiales*, such as *Eubacterium* sp. and *Flavonifractor* sp., and *Eggerthella* spp., have been reported to be able to initiate the metabolism of flavanol-3-ols (Jin & Hattori, 2012; Kutschera, Engst, Blaut, & Braune, 2011; Wang et al., 2001). Although batch incubations with fecal microbiota are limited by substrate depletion and the accumulation of the end products, they are appropriate for comparison of the microbial consequences of exposure to different sources or doses of compounds, such as the cranberry and grape seed extracts used in this study.

In summary, this study reports for the first time the formation of phenolic acids and other metabolites after *in vitro* incubations of cranberry polyphenols with colon microbiota, to a relatively greater extent in comparison to polyphenols from other sources (i.e., grape seeds). These microbial-derived metabolites may play a key role in explaining the preventive effects of cranberry consumption against UTIs. Recently, the anti-adhesive activity against uropathogenic *E. coli* by some microbial-derived metabolites, such as catechol, benzoic acid, vanillic acid, phenylacetic acid and 3,4-dihydroxyphenylacetic acid, has been proven in cultures of epithelial bladder cells (González de Llano et al., 2014).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.061.

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