Title

Cranberries attenuate animal-based diet-induced changes in microbiota composition and functionality: a randomized crossover controlled feeding trial

Authors and affiliations

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Running tittle: Cranberries on microbiota and functionality

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Keywords

Clinical trial; cranberries; microbiota; polyphenols; bile acids; short-chain fatty acids

Abstract

Cranberries have multiple health effects but their impact on gut microbiota has not been examined in randomized controlled feeding trials. We evaluated the relationship between the microbiota and cranberries in the context of an animal-based diet. In a randomized, double-blind, cross-over, controlled design trial, 11 healthy adults consumed for 5 days each a control diet (animal-based diet plus 30 g/day placebo powder) and a cranberry diet (animal-based diet plus 30 g/day freeze-dried whole cranberry powder). The animal-based diet included meats, dairy products, and simple sugars. Stool, urine, and blood samples were obtained before and after each intervention phase. As compared to the pre-control diet, control diet modified 46 taxonomic clades, including an increase in the abundance of *Firmicutes* and decrease in *Bacteroidetes*. Moreover, it increased bacteria-derived deoxycholic acid and decreased acetate and butyrate in stool. As compared to the post-intervention phase of control diet, the cranberry diet modified 9 taxonomic clades, including a decrease in the abundance of *Firmicutes* and increase in *Bacteroidetes*. Further, the cranberry diet attenuated control diet-induced increase in secondary bile acids and decrease in short-chain fatty acids (SCFA), and increased urinary anthocyanins and bacterially derived phenolic acids. No changes were found in fecal trimethylamine and plasma cytokines. In conclusion, an animal-based diet altered the microbiota composition to a less favorable profile, increased carcinogenic bile acids, and decreased beneficial SCFA. Cranberries attenuated the impact of the animal-based diet on microbiota composition, bile acids, and SCFA, evidencing their capacity to modulate the gut microbiota.

Keywords

Clinical trial; cranberries; microbiota; polyphenols; bile acids; short-chain fatty acids

Chemical compounds

Trimethylamine (PubChem CID: 1146); trimethylamine *N*-oxide (PubChem CID: 1145); lithocholic acid (PubChem CID: 9903); deoxycholic acid (PubChem CID: 222528); acetic acid (PubChem CID: 176); propionic acid (PubChem CID: 1032); butyric acid (PubChem CID: 264).

1. Introduction

A higher consumption of plant relative to animal food based diets is associated with a lower risk of all-cause mortality, particularly from cardiometabolic diseases [1]. In addition to being rich in nutrients, plant foods are a source of bioactive phytochemicals and fiber, all of which have beneficial effects on human health [2]. A growing body of evidence illustrates that the nutritional value of foods is subject to the influence of the microbiota in the human gut [3]. Inversely, food shapes gut microbiota composition [4]. The complex interaction between foods and the microbiota can modulate human health both positively and negatively. Gut bacteria have the ability to metabolize inaccessible dietary components, synthesize essential vitamins, provide protection from pathogens, and regulate the immune system [3]. However, dysbiotic composition and decreased diversity and richness of the gut microbiota are also linked to several chronic diseases, including atherosclerosis, hypertension, kidney disease, and type 2 diabetes [5-7]. Thus, the gut microbiota has been considered an endocrine organ that has a dramatic impact on the health of the host [6].

Secondary bile acids, short-chain fatty acids (SCFA), and trimethylamine (TMA) are well-studied examples of microbiota-biosynthesized molecules [6, 8]. Bile acids are molecules that facilitate digestion and lipid absorption in the small intestine but they also act as signaling molecules. The liver synthesizes primary bile acids (cholic acid and chenodeoxycholic acid), which are then dehydroxylated by intestinal bacteria to secondary bile acids (deoxycholic acid and lithocholic acid) [9], which are considered possible carcinogens [8]. SCFA, a subset of saturated fatty acids containing six or fewer carbon molecules, are the main metabolites produced by colonic anaerobic bacteria following the fermentation of dietary fiber, and they have been shown to exert beneficial effects on health [3, 10]. Acetate, propionate, and butyrate are the

primary SCFAs in the gut, constituting over 95% of the total production [11]. TMA is an organic compound generated by gut bacteria from choline, phosphatidylcholine, and carnitine (all three found at high concentrations in red meat, eggs, milk and fish) [12, 13]. Once absorbed and transported to the liver, TMA is oxidized to trimethylamine *N*-oxide (TMAO), which is considered a potential promoter of atherosclerosis and cardiometabolic diseases [6].

The wide variation in the gut microbiota between individuals is a result of modulations of many dietary, environmental, physiological, and lifestyle factors. Clinical evidence has illustrated that diet can alter the composition and diversity of human gut microbiota within 24 hours [14], and also modify their metabolic activity [15]. Cranberries (*Vaccinium macrocarpon*) are fruits associated with multiple health benefits [16, 17], mainly attributed to (poly)phenolics, including proanthocyanidins, anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, and terpenes. Among the 20 most commonly consumed fruits in the American diet, cranberries have the highest total phenol content [18]. These (poly)phenolics contribute to their noted antibacterial [19], antiviral [20], anti-tumorigenic [21], antiangiogenic [22] and antioxidant activities [18]. Following ingestion of cranberries, a small proportion of phenolic acids and flavonoids is bioavailable and have physiological consequences [23]. Those that remain unabsorbed in the upper gastrointestinal tract can have a reciprocal relationship with the microbes in the large intestine, a result of anti-microbial and bacteriostatic activities of (poly)phenolics [24] and metabolic activity of gut bacteria towards (poly)phenolics [25, 26]. Emerging evidence including studies in animal models [27] and a recent prospective clinical trial [28] showed that cranberries had an impact on gut microbiota. Nevertheless,randomized controlled clinical trials with a well-controlled diet that diminishes the confounding effect of individual background diet are needed to confirm these observations [29].

In the present work, we examined the impact of cranberry constituents on the bacteria profile and bacterially derived products, including secondary bile acids, SCFAs, and TMA, in a small scale, randomized, crossover, blinded, and controlled feeding trial. In the framework of the study, we also aimed to confirm the absorption of cranberry anthocyanins, flavonoids, and phenolic acids.

2. Materials and methods

2.1. Study subjects and design

We conducted a randomized, placebo controlled, double-blind, crossover study in 11 healthy subjects (7 males, 4 females) with a regular bowel movement (≥3 times/week) and a mean age of 39.2 ± 12.3 years (ranging from 25 to 54 years). A detailed description of the inclusion and exclusion criteria is available in the **Supplementary Material 1.** Baseline characteristics of participants are presented in **Table 1**. The trial included two 5-day intervention phases with a 2-week washout period between phases. After enrollment, eligible subjects were instructed to consume their habitual diet during the one-week run-in period. Subsequently, subjects were randomly assigned to receive either cranberry diet (basal diet plus 30 g/day of freeze-dried whole cranberry powder) or control diet (basal diet plus 30 g/day of matched placebo powder). The details on the interventional diets are described in section 2.2. During the 2-week washout period, the subjects were instructed to consume their habitual diet, similar to the run-in period. During the whole study, subjects were required to visit the study site for 12 times over 8 weeks (Study timeline is depicted in **Fig. S1**).

A total of 4 stool, urine, and blood samples (one each before and after dietary intervention phase) were collected from each subject for laboratory analyses. Subjects were provided with disposable

commode specimen containers (Claflin Medical Equipment, Warwick, RI) for collecting stool samples. The whole evacuated stool was placed in a sealed bag, then in a cooler with ice packs, and transported to the study site within 18 hours after the evacuation. Subjects were instructed to not stop consuming the study meals until the first bowel movement of the post 5-day feeding intervention. Morning spot urine samples were provided by volunteers during the study visits, Urine samples were collected in 500-mL polypropylene containers and were stored at 5°C prior to being aliquoted into cryovials for storage at -80°C. Fasted venous blood samples were collected from a hand or arm vein. Blood was collected into 10 mL tubes containing EDTA and centrifuged (1690 *g*, 15 min, 4 °C). The plasma samples were immediately separated and frozen at -80 °C until analyses. The trial was approved by the Institutional Review Board of Tufts University Health Sciences Campus and Tufts Medical Center. All participants signed a written informed consent agreement before any study conducts were performed. This study was registered with the public registry ClinicalTrials.gov (NCT02677649).

2.1. Anthropometric measures

Body weight, height, BMI, and waist and hip circumferences were measured according to standardized procedures [30] and as detailed in **Supplementary Material**. The same apparatus and equipment were used in all subjects.

2.2. Control diet and cranberry diet

Both interventional diets were prepared in the kitchen of the Metabolic Research Unit at Tufts University and packed frozen study meals were provided to subjects to consume at home or work. The basal diet was comprised of meats, dairy products, simple sugars, and stevia, and the diet was formulated based on the David *et al.* study [15], in which an animal-based diet significantly altered microbiota profile. Control diet and cranberry diet contained 30 g of placebo

149 powder and freeze-dried whole cranberry powder, respectively, whose nutrition composition is
150 presented in **Table S1**. An example of a one-day menu and its corresponding nutrient
151 composition are presented in **Tables S2 and S3,** respectively. The 30-g dosage was selected in
152 order to provide sufficient amounts of flavonoid and proanthocyanidins that were comparable to
153 the average flavonoid and proanthocyanidins intake at 157 mg and 95 mg/day, respectively, in
154 the United States [31, 32]. The caloric content of each diet was adjusted to each subjects' caloric
155 need for body weight maintenance using the Harris-Benedict equation and adjusting by activity
156 factor.
157 The freeze-dried whole cranberry powder was produced from a blend of cranberry varieties
158 similar to the market reality at the time of production (56% Stevens, and 11% each of Ben Lear,
159 Grygleski, Pilgrim, and HyRed). The berries were individually frozen after harvest, freeze-dried,
160 and ground into powder form. Silicon dioxide (3% total volume of powder) was added as an
161 anti-caking agent. The processing and packaging facilities are compliant with U.S. Food and
162 Drug Administration regulations. The placebo powder was produced from a blend of water,
163 maltodextrin (CPC Maltrin M-180), citric acid, artificial cranberry flavor (Lorann oils), fructose,
164 red color (Lorann oils), and grape shade (Esco Foods) that was then freeze-dried. The placebo
165 was manufactured by the United States Department of Agriculture (USDA), Agricultural
166 Research Service, Western Regional Research Center, Healthy Processed Foods pilot plant in
167 Albany, CA. Tea and other plant-based beverages were not allowed during the intervention
168 phases.

169 **2.3. Gut microbiota analysis**

170 Gut microbiota in the fecal samples was determined by 16S rRNA pyrosequencing in the
171 Phoenix laboratory of the Tufts Medical Center. Fecal DNA was extracted by enzymatic

digestion and bead-beating steps, followed by the use of a QIAamp Stool DNA Mini Kit (Qiagen). 16S rRNA gene amplicons were generated from the extracted DNA using PCR with a barcoded primer set targeted to the V4 variable region [33]. Amplicons were pooled in equimolar amounts and then purified for 250 bp paired end sequencing using an Illumina MiSeq platform (Illumina Inc.). A custom pipeline was used for sequence data analysis, using the Quantitative Insights Into Microbial Ecology (QIIME) software v1.8.0 [34]. After quality filtering, paired-end sequences were concatenated and demultiplexed. Closed reference Operational taxonomic units (OTU) at 99% similarity were assigned using Greengenes v13_5 reference database [35] and USEARCH v6.1 [36]. Alpha diversity indices Chao 1, Shannon, and Sipmson's were calculated using QIIME. Beta diversity was evaluated using unweighted and weighted UniFrac metrics through QIIME. Differences in OTU abundance were analyzed by linear discriminant analysis (LDA) effect size (LEfSe) method according to Segata *et al.* [37]. LEfSe analysis shows those OTUs that were significantly differentially abundant between comparisons, ranked by effect size. The bacterial taxa with significant differences were used to build the LDA model and to estimate its effect as a discriminant feature. The threshold used to consider a discriminative feature for the logarithmic LDA score was set to >2 (further details are provided in subheading *2.6. Statistical analysis*).

2.4. Analysis of (poly)phenolics, bile acids, SCFA, and TMA

2.4.1. Urinary (poly)phenolics and anthocyanins

Urinary phenolic acid and flavonoids were determined by a routine HPLC-electrochemical detection method established in our laboratory as previously described [38]. Data were normalized by creatinine concentrations and are expressed as μg/mg creatinine.

Anthocyanins in urine were quantified by a LC-MS/MS as previously described [39]. Data were normalized by creatinine concentrations and are expressed as pg/mg creatinine.

2.4.2. Fecal Bile acids

Individual bile acids, including lithocholic, cholic, deoxycholic, and chenodeoxycholic acids, in feces were determined by LC-QTOF/MS. Stool samples were freeze-dried and stored at -80°C before the analysis. The sample preparation method requires a two-day procedure and included overnight extraction from freeze-dried samples using a mixture of chloroform and methanol, followed by a multi-stage purification process. In brief, a 10.0 mg aliquot of sample was weighted and then spiked with 50 µL of internal standard solution (containing D_4-cholic acid and D_4-lithocholic acid in methanol). The resulting mixture was incubated in 6 mL of chloroform:methanol (2:1) solution overnight in a refrigerator. After centrifugation for 10 min at 4,000 *g*, the supernatant was transferred, dried under nitrogen gas, reconstituted with 1 mL of mobile phase (10 mM ammonium acetate and 0.1% ammonium hydroxide in methanol at pH 9), and then diluted 100 times (two sequential 1:10 dilutions) for LC-QTOF/MS analysis. For further analytical details, see **Supplementary Material**. Data were normalized by dry weight and are expressed as mg/g.

2.4.3. Fecal SCFA

Fecal SCFA were measured by a QTRAP 5500 LC-MS/MS method as previously described. The method employs a derivatization with 3-nitrophenylhydrazine and measures the SCFA in stool samples [40]. Data are expressed as µmol/g.

2.4.4. TMA and TMAO

TMA and TMAO in three different matrixes (plasma, urine and stool) were determined by a QTRAP 5500 LC-MS/MS method as previously described [41].

2.4.5. Water content and pH

Water content in feces is the weight loss of wet stool samples after they were freeze-dried. It was calculated using the following formula:

$$\text{Water content} = \frac{mass\ of\ the\ wet\ sample - mass\ of\ the\ dried\ sample}{mass\ of\ the\ wet\ sample}\ x\ 100$$

pH value in feces was determined using a pH meter.

2.4.6. Plasma cytokines

Inflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α in plasma were determined following manufacturer's instructions using a Meso Scale Discovery Multiplex kit (Rockville, MD). Data are expressed as pg/mL.

2.5. Sample size and power analysis calculation

The sample size for this human trial (n=11) was estimated based on the effect of freeze-dried whole cranberry powder on the animal-based diet induced increase in bile tolerant bacteria [15] and on the increase in *Enterococcus*, *Bifidobacterium*, *Eggerthella lenta*, and *Blautia coccoides–Eubacterium rectale* groups [42]. There are no data in the literature illustrating the effect of freeze-dried whole cranberry powder on bile tolerant bacteria for power calculation, but our sample size is comparable to the number of subjects in the studies reported by David *et al.* [15] and Queipo-Ortuño *et al*. [42], respectively.

2.6. Statistical analysis

Statistical analyses of data generated during the study were performed using the current version of the SAS statistical software package (SAS Institute Inc., Cary, NC). A Mixed ANOVA model, including treatment, sequence, and period as the independent variables, was used to determine statistically significant differences, followed by Tukey's Honest Significant Different (HSD) multiple comparison tests. This model was employed for the statistical analysis of

(poly)phenolics, bile acids, SCFA, TMA, pH, water content, and inflammatory cytokines. The effect of the control diet was evaluated comparing in *post-hoc* analysis differences between values at baseline versus values after control diet. The cranberry effect was evaluated comparing in *post-hoc* analysis differences between the values after control diet versus the values after cranberry diet. A p-value ≤ 0.05 was considered statistically significant. Pearson's and Spearman's correlation coefficients (r) and p-values were used to determine correlations between variables. Normality of continuous variables related to microbiota abundance and biochemical parameters was assessed and, if required, data were log transformed. P-values were adjusted for multiple comparisons to control the false discovery rate, generating p-adjusted values (also known as q-values). Correlation analyses were carried out in R Microbiome package and graphs were plotted with GraphPad Prism. Adonis (analysis of variance) and Anosim (analysis of similarity) tests were performed using Vegan package in R on the post-interventional data. Principal Coordinate Analysis (PCoA) was analyzed to illustrate beta-diversity using Phyloseq package in R.LEfSe consists of an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features (genes, pathways, or taxa) characterizing the differences between two or more biological conditions. This algorithm uses firstly the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance with respect to the class of interest, then the (unpaired) Wilcoxon rank-sum test to assess biological consistency and finally a linear discriminant analysis estimates the effect size of each differentially abundant feature [37].

3. Results

3.1. Participants baseline characteristics

Table 1 presents the baseline characteristics of the 11 healthy volunteers that completed the study and the corresponding values grouped by sex. Of these subjects, 7 were male and 4 were female, their average age was 39.2 ± 12.9 years and their average BMI was 22.2 ± 2.0 kg/m^2. Their baseline waist circumference was 81.5 ± 8.3 cm and their hip circumference was 93.7 ± 4.3 cm. Diastolic blood pressure was 81.4 ± 7.9 mm Hg, systolic blood pressure was 116.5 ± 12.6 mm Hg and the heart rate was 70 ± 13 beats per minute.

3.2. Gut microbiota analyses

3.2.1. Diet effects on microbiota diversity

Neither the control diet or the cranberry diet altered microbiota diversity. Within sample diversity (alpha diversity) remained unaltered, as no differences in bacterial richness and evenness were found using three different diversity metrics: Chao 1 (total number of species), Shannon (heterogenecity), and Simpson's index (similarity) (**Fig. S2**). Between environment/community diversity (beta diversity) was not altered, as no differences in weighted and unweighted variants of UniFrac were found. PCoA plot (**Fig. S3**) also showed cranberry did not affect beta diversity of the fecal microbiota as compared to control. The results of Adonis (R^2 = 0.0245, P = 0.962) and Anosim (R = -0.1002, P = 0.998) tests on the post-interventional microbiome further confirmed that diversity of the fecal microbiome was not altered by the addition of cranberry to the control diet.

3.2.2. Diet effect on relative phylum abundance

The control diet altered the relative abundance of fecal bacteria with a decrease in the gram-negative *Bacteroidetes* (p = 0.018) and an increase in gram-positive *Firmicutes* (p = 0.023), to

286 the extent that *Bacteroidetes* was the most abundant phylum before the diet and *Fimicutes*
287 became the most abundant after the diet. No differences were found in the relative abundance of
288 the phyla *Actinobacteria*, *Proteobacteria* nor *Verrucomicrobia* between groups (**Fig. 1**).
289 The addition of cranberries to the basal diet reversed the effect of the control diet on the relative
290 abundance of phyla, as shown by an increase in *Bacteroidetes* ($p = 0.032$) and a decrease in
291 *Firmicutes* ($p = 0.038$).

292 ***3.2.3. Diet effect on the overall relative abundances***

293 The control diet altered the relative abundance of gut microbiota. LEfSe analysis comparing the
294 relative abundances of microbiota at baseline vs. after 5 days of control diet identified 46 altered
295 bacterial clades. Specifically, the control diet induced an increase in 35 bacterial clades,
296 including 1 phyla (*Firmicutes*), 3 classes, 4 orders, 7 families, 15 genera and 5 species (**Fig. 2**).
297 The highest diet-induced increases in relative abundance were in the phyla *Firmicutes*, the class
298 *Clostridia* and the order *Clostridiales* (LDA >4.8). Furthermore, the control diet induced a
299 decrease in 11 bacterial clades, including 1 phyla (*Bacteroidetes*), 2 classes, 2 orders, 3 genera,
300 and 3 species. The highest diet-induced decrease in the relative abundance were in the phyla
301 *Bacteroidetes*, the class *Bacteroidia* and the order *Bacteroidales* (LDA >4.8) (**Fig. 2**).

302 A significant effect of cranberry diet on the relative abundance of fecal bacteria was observed, as
303 LEfSe analysis comparing relative abundances after control diet versus after cranberry diet
304 identified 9 differentially abundant taxonomic clades with LDA score larger than 2. Specifically,
305 after cranberry diet, there was an increase in the relative abundance of the phylum *Bacteroidetes*,
306 the class *Bacteroidia*, and the order *Bacteroidales*, as well as an increase in the genera
307 *Lachnospira* and *Anaerostipes.* Further, the phyla *Firmicutes*, the class *Clostridia*, the order

Clostridiales and the genus *Oribacterium* had lower relative abundances after the cranberry diet, compared to the values after the control diet (**Fig. 3**).

3.3. Urinary anthocyanins

The urinary level of four major cranberry anthocyanins (cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside, and peonidin-3-arabinoside) and two minor cranberry anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) are depicted in **Fig. 4A** and **S4**, respectively. Despite large inter-individual variability, all major cranberry anthocyanins were significantly higher in morning spot urine at the end of cranberry phase (CRA post) compared to those before the cranberry intervention (CRA pre) and to those before and after control, which all had values generally close to zero (**Fig. 4A**). In the case of minor cranberry anthocyanins, no significant differences were found between groups (**Fig. S4**).

3.4. Urinary phenolic acids and flavonoids

The urinary levels of 15 phenolic acids and flavonoids were measured before and after the two different diets. A cranberry-induced treatment effect was observed as the concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) and its *ortho*-methylated metabolite 4-hydroxy-3-methoxyphenylacetic acid (Homovanillic acid; HVA) were 160 and 72% higher, respectively, at the end of the cranberry phase than those at the end of the control phase ($p < 0.01$) (**Fig. 4B**). No other differences between treatments were found for the other phenolic acids and flavonoids measured: protocatechuic, genistic, 3-hydroxybenzoic, 4-hydroxyphenylacetic, vanillic, 4-hydroxybenzoic, caffeic, *p*-coumaric, ferulic, and sinapic acids, and catechin, epicatechin, quercetin, and myricetin (**Fig. S4**).

3.5. Fecal bile acids

Among the bile acids measured, none of the primary (lithocholic and cholic), and only the secondary bile acids (lithocholic and deoxycholic) were detected. Both secondary bile acids followed a similar pattern, but the effects were more remarkable for deoxycholic acid, whose concentrations after 5 days of control diet increased by 268%, compared to the pre-control diet values (**Fig. 5A**). Interestingly, when this same basal diet was supplemented with whole cranberry powder, the levels of these secondary bile acids did not increase and were statistically lower than those after the control diet (**Fig. 5A**).

3.6. Fecal SCFA

The levels of SCFA quantified in the stools are depicted in **Fig. 5B**. No differences were found in the levels of propionic acid between groups. The levels of acetic ($p < 0.005$) and butyric acids ($p < 0.01$) decreased by 40 and 48%, respectively, after the consumption of the control diet for 5 days, compared to pre-control diet values. The addition of cranberry powder to the basal diet attenuated the magnitude of the decreases to the extent that no differences were found before and after the diet.

3.7. TMA and TMAO

The levels of TMA and TMAO in plasma, urine and stool are summarized in **Fig. S6**. No significant changes between the 4 groups (before and after each intervention) were detected in the levels of TMA and TMAO. No TMAO was detected in feces. This observation is in agreement with the literature [43].

3.8. Water content and pH

Fecal water content and pH values were measured as these parameters can be modified by diet [44]. Moreover, the measurement of fecal water content allows the normalization of the

353 concentration of biomarkers measured in stool samples when significant differences in water
354 content are found.
355 Fecal pH values increased significantly after the control diet, but the addition of cranberry
356 powder did not change the pH (**Fig. 6A**). Fecal water content presented a high inter-individual
357 variability and no diet-induced differences were noted.

358 **3.9. Inflammatory biomarkers**

359 Circulating levels of IFN-γ, IL-1β, IL-6, and TNF-α after and before each diet are summarized in
360 **Fig. S5**. No differences on circulating cytokines were found as a consequence of the animal-
361 based diet or by the addition of cranberry.

362 **3.10. Correlation analyses**

363 Correlation analyses between different variables of taxonomic data from 16S rRNA sequencing
364 and biochemical parameters revealed some correlations that remained significant following the
365 false discovery rate correction. The most relevant findings are the positive correlation between
366 the levels of deoxycholic acid and the abundance of *Fusobacterium* (Spearman r = 0.74; p
367 <0.0005; adjusted p <0.05) (**Fig. 6B**), the negative correlation between deoxycholic acid and the
368 abundance of *Anaerostipes* (Spearman r = -0.50; p <0.001; adjusted p <0.05) (**Fig. 6C**), the
369 negative correlation between fecal pH values and the levels of butyric acid in stool (Pearson r = -
370 0.75; p <0.0001; adjusted p <0.001) (**Fig. 6D**), and the negative correlation between fecal pH
371 values and the levels of acetic acid in stool (Pearson r = -0.63; p <0.0001; adjusted p <0.001)
372 (**Fig. 6E)**.

373 **4. Discussion**

374 In this small scale randomized controlled feeding study, we found that an animal-based diet
375 (comprising meats, dairy products, and simple sugars) altered in a short-period (5 days) the

microbiota relative abundance as well as the level of microbiota-derived compounds. Moreover, the addition of cranberries to this diet restored part of the alterations in microbiota composition and functionality towards a healthier profile, and increased urinary levels of anthocyanins and phenolic acids.

Although it has been proposed that the beneficial effects of fruits in general [45] and polyphenols in particular [46] could be mediated through their interaction with gut microbiota, there are only a limited number of clinical studies evaluating the relationship between polyphenols and microbiota composition and bacterial functionalities, such as palm dates [47], cocoa flavanols [48], blueberries [49] and wine [42, 50].

In our study, the control diet decreased the relative abundance of the gram-negative *Bacteroidetes* and increased the gram-positive *Firmicutes*, with a corresponding increase in the *Firmicutes/Bacteroidetes* ratio. This ratio has consistently been found to associate positively with weight gain and inversely with weight loss [51], and it has been proposed as a biomarker of obesity [52]. In our study, the cranberry diet reversed the control diet effects on the abovementioned changes, suggesting constituents in cranberries, yet to be fully identified could counteract the animal-based diet induced changes in the fecal microbiome and potential detrimental sequelae. An increase in *Bacteroidetes* and decrease in *Firmicutes*, similar to the observed after the cranberry diet, has been observed with weight loss on two types of low-calorie diets (fat- and carbohydrate- restricted diets) [53], after red wine intake over a 30-day period in patients with metabolic syndrome [50], and in a recent prospective study with sweetened dried cranberries [28].

At the genus and species level, the control diet significantly increased the relative abundance of several microorganisms, some of which linked to detrimental effects on human health, like

Solobacterium moorei (associated with bacteraemia and colorectal cancer) [54], *Ruminococcus* and *Ruminococcus gnavus* (inflammatory bowel disease [55]), *Clostridium clostridioforme* (infection and antibiotic resistance [56]), *Dorea* and *Dorea formicigenerans* (obesity [57]), and the genus *Bilophila* (bile acid metabolism [15] and associated with colitis in mice [58]), *Gemella* (opportunistic pathogen related to infections [59]) and *Actinomyces* (infection [60]). On the other hand, the control diet decreased the relative abundance of some bacteria linked to beneficial health effects like the genus *Lachnospira* (associated with vegetable intake [61]) and *Anaerostipes* (butyrate production [62]) as well as the species *Ruminococcus bromii* (resistant starch degradation [63]).

The cranberry diet reversed, in part, the alterations induced by control diet on microbiota abundances. Indeed, cranberry constituents not only shifted the abovementioned abundances in *Firmicutes* and *Bacteroidetes* but also increased the abundance of *Lachnospira* and *Anaerostipes* (which contribute to SCFA production [62]) and decreased that of *Clostridia* and *Oribacterium*. It is worth noting that the 9 bacterial clades that were modified by cranberry diet altered the relative bacteria abundances in the opposite direction of the control diet to the extent that the effect of the latter was abolished. However, we did not see any changes in the beneficial genera *Bifidobacterium,* whose abundance has been reported to be increased by a wild blueberry drink [49], cocoa flavanols [48], and red wine polyphenols [42, 50]. Further, the increase in *Lactobacillus* was noted by cocoa flavanols [48] and red wine polyphenols [50] but not in our study. We speculate that the extreme basal diet administered in our trial might limit the favorable effects of cranberry constituents on the growth of probiotic bacteria, although the treatment and washout durations could also be contributing factors as recent studies in mice have shown that diet switching can result in lasting effects on the gut microbiota [64-66].

In the previously mentioned prospective study evaluating the effect of sweetened dried cranberry on fecal microbiome in healthy people [28], the authors did not observe treatment-induced differences in the β-diversity of the fecal microbiome, but noted a cranberry-induced decrease in the *Firmicultes/Bacteroidetes* ratio. These results are in line with our observations despite the differences in administration form (freeze-dried cranberry powders vs. sweetened dried cranberries), dosage (30 vs. 42 g), duration (5 days vs. 2 weeks), and design (randomized crossover controlled vs. prospective). Interestingly, an increase in the *Akkermansia* relative abundance after the sweetened dry cranberry treatment was noted in Nell *et al.* study [28], but not in our study. On the contrary, we noted the increased relative abundance of the genera *Anaerostipes* and *Lachnospira* after the cranberry diet. In order to (1) confirm the intake of cranberries by the volunteers, (2) assess the bioavailability of cranberry (poly)phenols, and (3) study potential correlations between cranberry constituents and microbiota-produced biomarkers, we measured urinary levels of anthocyanins, flavonoids and phenolic acids. The levels of urinary anthocyanins found after the cranberry diet are consistent with the anthocyanin profile in cranberries. The 4 major cranberry pigments in the descending order of their contents are peonidin-3-galactoside, cyanidin-3-galactoside, peonidin-3-arabinoside, and cyanidin-3-arabinoside, whereas the 2 minor anthocyanins are peonidin-3-glucoside and cyanidin-3-glucoside [67, 68]. The urinary levels of four major pigments were statistically higher after the cranberry diet, confirming our previous observation in volunteers after the intake of cranberry juice [23, 69]. However, the levels of minor anthocyanins did not reach statistical significance, mainly attributable to a wide inter-individual variation in polyphenol pharmacokinetics. Globally, these data provides solid evidence that cranberry anthocyanins are bioavailable, and the major qualitative advantage compared to previous reports, is the controlled feeding design of

445 the present study. The measurement of 15 phenolic acids and flavonoids in urine before and after
446 both dietary interventions revealed a cranberry-mediated effect. We noted that the levels of 3,4-
447 dihydroxyphenylacetic acid and its analogue (4-hydroxy-3-methoxyphenylacetic acid) were
448 higher at the end of the cranberry diet. It is worth noting that, according to *in vitro* fermentation
449 studies with human fecal bacteria, these two phenolic acids are microbial metabolites derived
450 from cranberry procyanidins [25, 70].
451 In order to provide insights in the connection between the diet-induced microbiota alterations
452 and cardiometabolic and chronic diseases, we measured three families of compounds that are
453 produced by the gut microbiota and that are linked to specific pathologies: (1) secondary bile
454 acids (implicated in carcinogenesis [71]), (2) SCFA (known for their effects on suppressing
455 inflammation, obesity, carcinogenesis, amongst others [10, 72]), and (3) TMA, whose further
456 hepatic oxidation generates TMAO (which associated with atherosclerosis and cardiovascular
457 disease) [73, 74].
458 The exclusive detection of the secondary bile acids (lithocholic acid and deoxycholic acid) are in
459 agreement with the fact that primary fatty acids are deconjugated and dehydroxylated by bacteria
460 in the colon [75]. The control diet-induced increase in the secondary bile acids supports potential
461 harms of excess consumption of red meats and processed meats because of their positive
462 association with increased risk of colon cancer [76] and other gastrointestinal cancers [8, 75].
463 Moreover, the unchanged secondary bile acid levels after the cranberry diet suggest a protective
464 effect of cranberry constituents on the gastrointestinal health by modulating the levels of
465 secondary bile acids. Interestingly, we noted that there was a strong negative association between
466 the levels of secondary bile acids and the relative abundance of *Anaerostipes*. It is worth
467 mentioning that the previous correlations only provide descriptive information regarding the

functional capacity of the gut microbiota and do not establish causality, evidencing the need of additional experiments using germ-free mice [77] or fecal microbiota transplants [78].

A significant decrease in acetic and butyric acid after the control diet was observed, and cranberry constituents mitigated this change to the extent that no differences were noted before and after the dietary intervention phase. Given that SCFA have an array of putative health benefits [11], our data suggests cranberry constituents could exert their beneficial effects by helping maintain SCFA production in the gut of people consuming diets low in fiber and high in animal meats and simple sugars.

The levels of TMA and TMAO did not differ between treatments. The lack of significance could be due to the high inter-individual variability observed and the small sample size.

Fecal pH values increased significantly after 5 days following the control diet, consistent with the literature in which increased meat consumption elevated fecal pH [79]. This is of relevance since fecal pH values that extend beyond the normal range are associated with the clinical course and prognosis of critically ill patients [80]. A strong negative correlation was observed between the fecal pH values and the concentrations of SCFA, being largest for butyric acid. This observation (together with the acidic character of these molecules and their high fecal concentrations) suggests the existence of a direct (or indirect) relationship between the levels of SCFA and the fecal pH values.

Our study has strengths and limitations. One strength is the use of a fully characterized freeze-dried whole cranberry powder and a matched placebo as standard reference materials. Their use enables comparability and replicability, and overcomes previous drawbacks to ensure consistency (e.g. confounding differences in the cranberry products and doses) [29]. Another strength of our study is the crossover design, in which each subject serves as his or her own

491 control. Thus, such a design provides two main advantages: (1) it minimizes interferences on the
492 study outcomes from confounding variables, such as diet, lifestyle, genetics, and others, on study
493 outcomes because those may arise when subjects are only tested in one study treatment and (2) it
494 allows detecting a smaller effect size with a reduced sample size. One limitation of our study is
495 the high cranberry dosage that we used (30 g), which was selected to meet daily average
496 flavonoid and proanthocyanidins intakes in the United States. Additional limitations of our study
497 include the small sample size (11 healthy individuals), inability to test sex specific differences,
498 and the extreme diet which, although it has been described in pastoralist and high-latitude
499 cultures [15], is not commonly consumed. Additional measurements relevant to cardiometabolic
500 outcomes such as plasma fasting glucose, lipids, or circulating lipopolysaccharide-binding
501 protein could have been measured to provide a better understanding of the impact of the
502 intervention. Another limitation is the lack of a detailed mechanistic understanding (at the
503 molecular level) of the relationship between microbiota, microbiota-produced metabolites
504 (secondary bile acids, SCFA, and trimethylamine), and the final impact on human health. Future
505 studies assessing whether metabolic capacity of the gut microbiota is modified by the study diets
506 are needed to establish causality. The results from this study provide a proof of concept that the
507 cranberry phytochemicals could attenuate the changes in microbiota and specific biomarkers
508 induced by an animal food-based diet. Nevertheless, we anticipate that such positive finding can
509 be extrapolated to the impact of foods rich in phytochemicals, particularly polyphenols, on shifts
510 of the gut microbiota and related biochemical and physiological to more healthful profiles.
511 Further, the design and results of this pilot study provide a basis for future studies with a larger
512 sample size to further substantiate the effect of phytochemical rich plant foods or dietary patterns
513 on the gut microbiota and related health outcomes. In a similar way, future studies with a larger

sample size will be necessary to establish to which extent the results observed in this study could be translated into clinical applications. Future studies with a larger sample size will be necessary to establish to which extent the results observed in this study could be translated into clinical applications.

5. Conclusions

In this randomized, cross-over, blinded, controlled feeding trial we observed that the consumption of an animal-based diet for a short period of time (5 consecutive days) altered the microbiota composition to a less favorable profile with the consequent alterations of microbiota-derived compounds (increasing the levels of carcinogenesis-related deoxycholic acid, and decreasing the levels of beneficial SCFA). Cranberry constituents modified the impact of the animal-based diet on microbiota composition and prevented the animal-based diet-induced increase in secondary bile acids and decrease in SCFA, evidencing their capacity to modulate the gut microbiota.

Conflict of interest

The authors declare that there is no conflict of interest.

Contributors

CYOC conceived and designed the trial. NRM and JL performed the experiments and data collection. JRM and CYOC analyzed the data. JRM drafted the first version of the manuscript. NM, RT and CYOC contributed to the discussion and reviewed the manuscript. All authors read and approved the manuscript.

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Figure captions

Fig. 1. Taxonomic profile of 5 major bacterial phyla in study participants before (pre) and after (post) consuming control diet (CON) or cranberry diet (CRA) for 5 days.

Fig. 2. Linear discriminant analysis score plot from linear discriminant analysis effect size (LefSe) analysis of the gut microbiota composition before (pre) and after (post) the animal-based diet. Positive LDA scores indicate bacterial clades with higher abundance at baseline (green bars) whereas negative LDA scores indicate bacterial clades with higher abundance after the control diet (red bars).

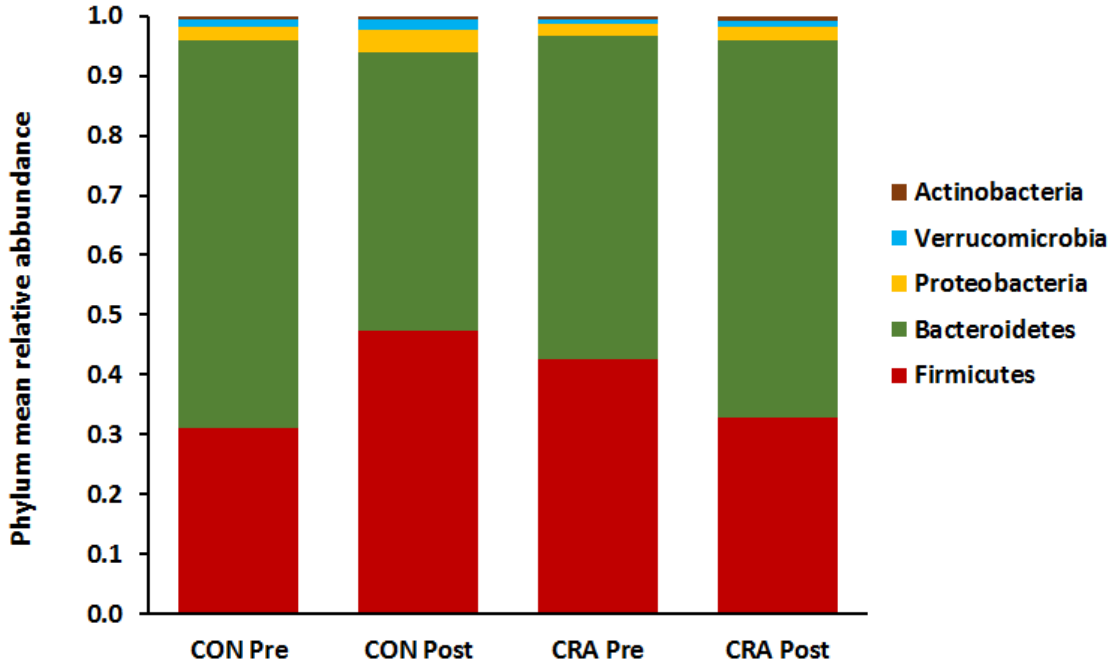
Fig. 3. Cladogram from linear discriminant effect size (LEfSe) analysis of stool samples after study participants consumed control diet (CON) or the cranberry diet (CRA) for 5 days. Positive LDA scores indicate bacterial clades with higher abundance after control diet (green) whereas negative LDA scores indicate bacterial clades with higher abundance after the cranberry diet (red).

Fig. 4. Urinary levels of major anthocyanins (**A**), and phenolic acids (**B**) before (pre) and after (post) consuming control diet (CON, closed circles) or cranberry diet (CRA, open circles) for 5 days. *p <0.05; **p <0.01; ***p <0.005.

Fig. 5. Concentrations of secondary bile acids lithocholic acid and deoxycholic acid **(A)** and short-chain fatty acids **(B)** in feces before (pre) and after (post) consuming either control diet (CON) or cranberry diet (CRA) for 5 d. *p <0.05; **p <0.01; ***p <0.005.

Fig. 6. (**A**) Fecal pH values before (pre) and after (post) study participants consumed control diet (CON) or the cranberry diet (CRA) for 5 days. Correlations between deoxycholic acid and *Fusobacterium* (**B**), deoxycholic acid and *Anaerostipes* (**C**), Fecal pH and fecal acetic acid (**D**), and Fecal pH and fecal butyric acid levels (**E**). P and r values are based on either Spearman or

Pearson correlation tests, as indicated in the Figure. Adjusted p-values (also known as q-values) indicate the p-values after optimization using the false discovery rate approach. **P <0.01; ***P <0.005.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1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CON Pre
CON Post
CRA Pre
CRA Post
Actinobacteria
Verrucomicrobia
Proteobacteria
Bacteroidetes
Firmicutes

Fig. 1

Relative abundance differences before and after control diet

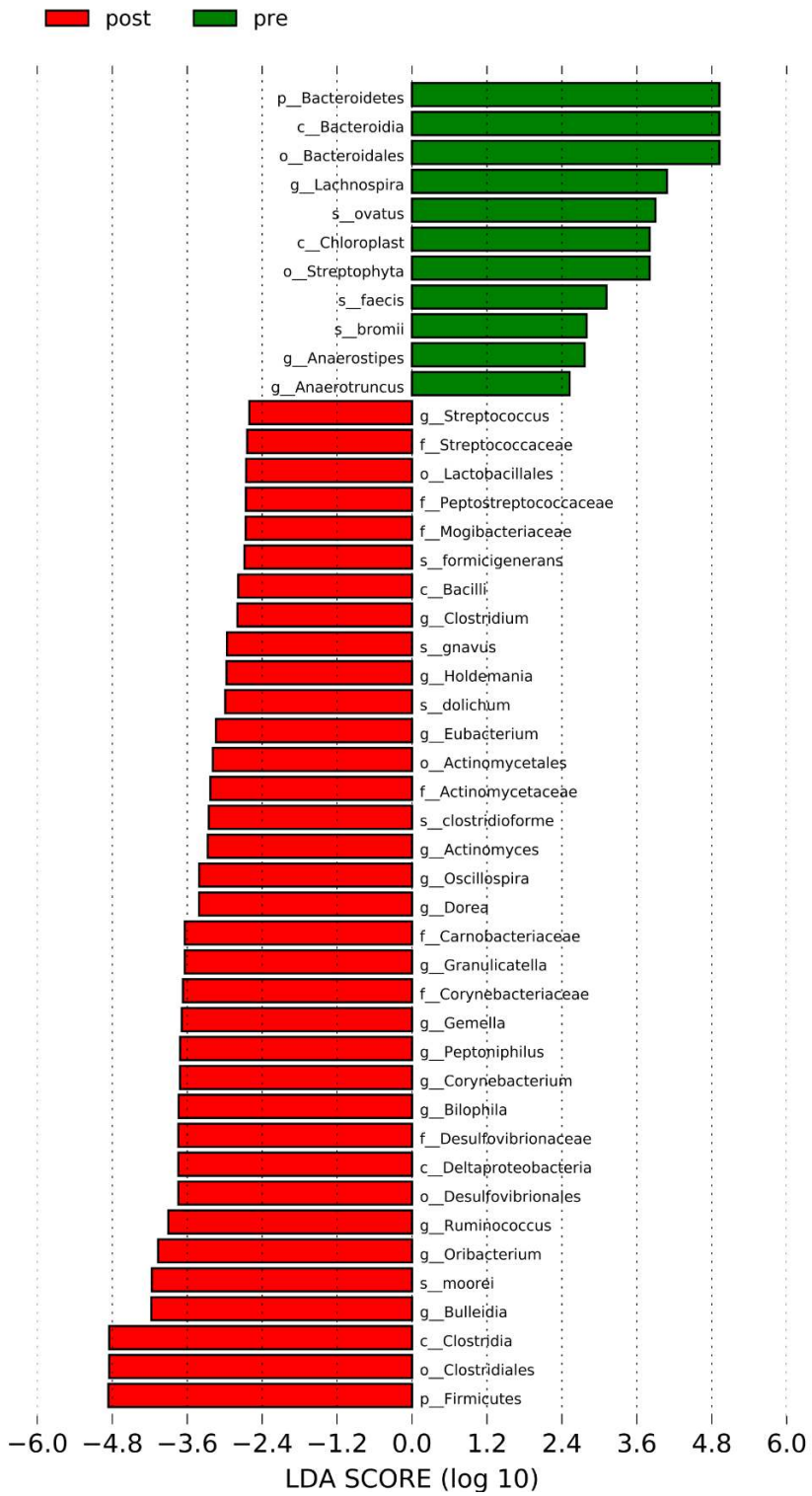

Fig. 2

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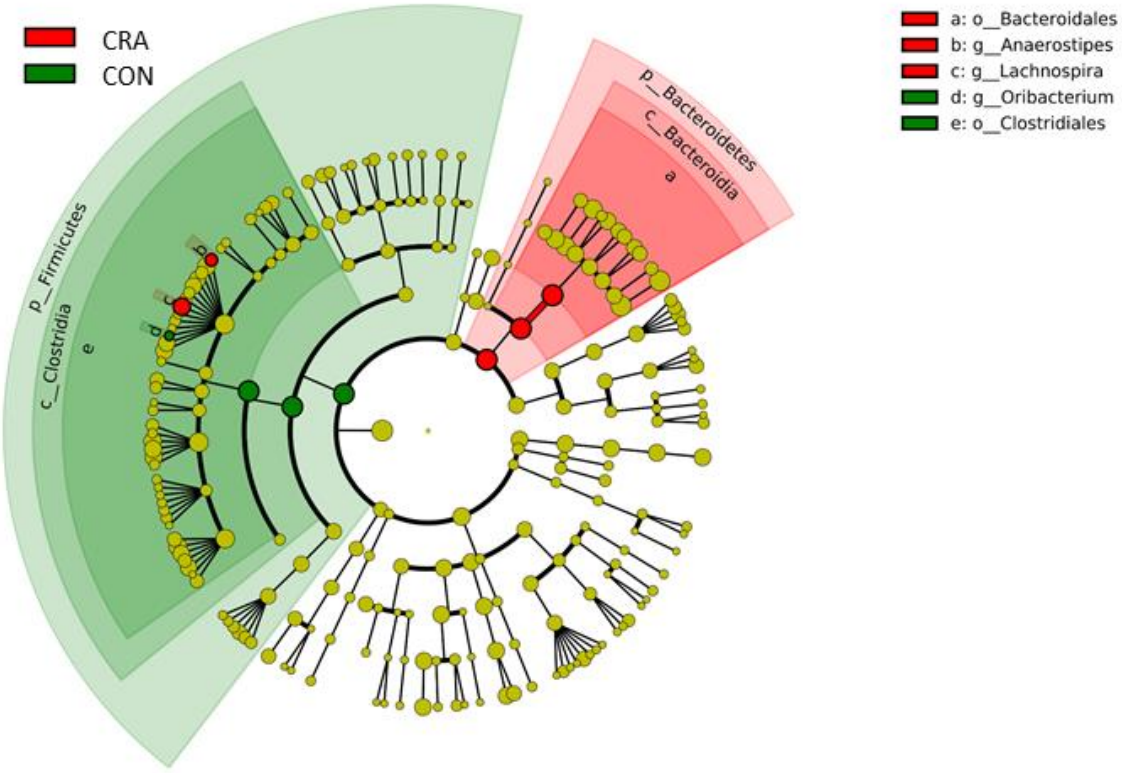
798 **Cladogram of relative abundance differences between cranberry diet and**
799 **control diet**



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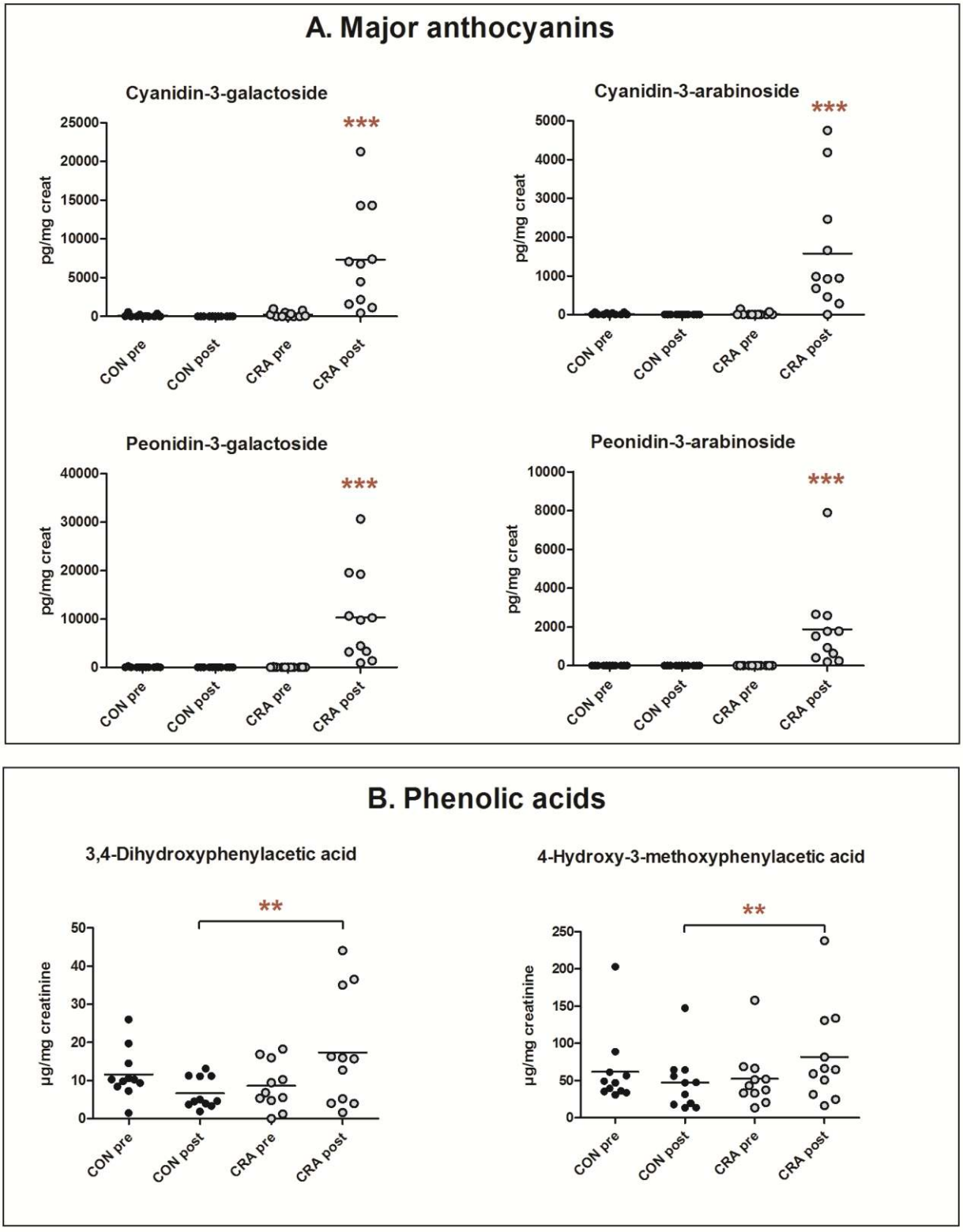
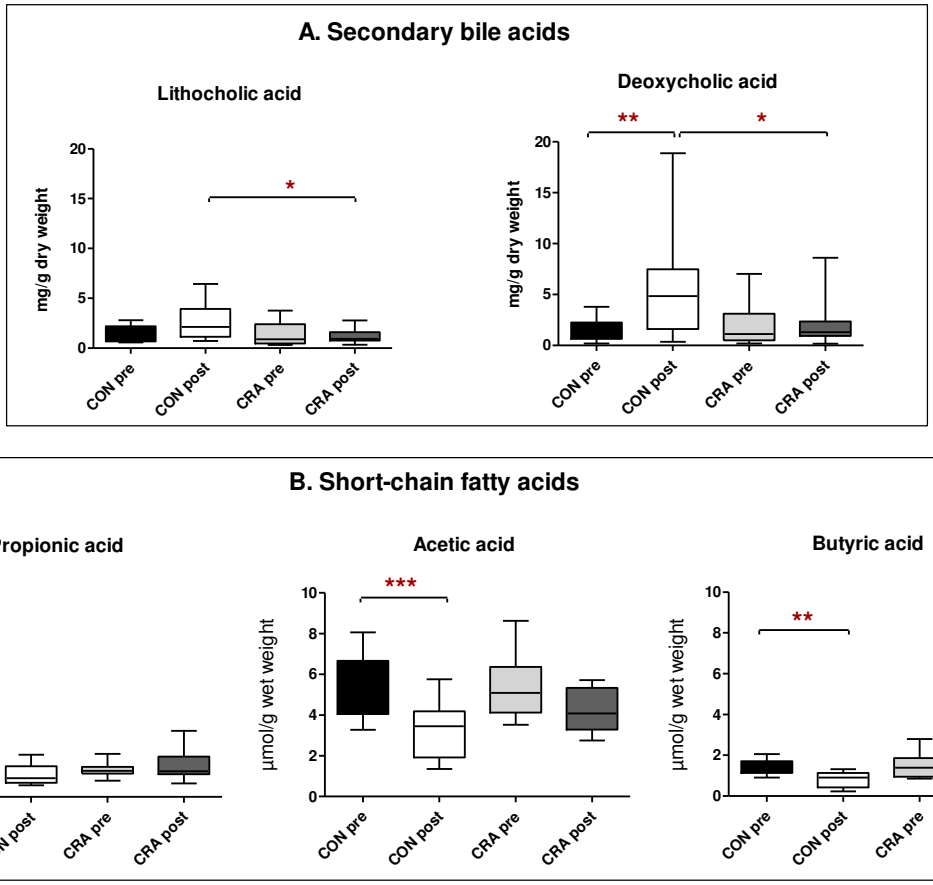
801 **Fig. 3**


A. Major anthocyanins
Cyanidin-3-galactoside
pg/mg creat
25000
20000
15000
10000
5000
0
***
CON pre
CON post
CRA pre
CRA post
Cyanidin-3-arabinoside
pg/mg creat
5000
4000
3000
2000
1000
0
***
CON pre
CON post
CRA pre
CRA post
Peonidin-3-galactoside
pg/mg creat
40000
30000
20000
10000
0
***
CON pre
CON post
CRA pre
CRA post
Peonidin-3-arabinoside
pg/mg creat
10000
8000
6000
4000
2000
0
***
CON pre
CON post
CRA pre
CRA post
B. Phenolic acids
3,4-Dihydroxyphenylacetic acid
µg/mg creatinine
50
40
30
20
10
0
**
CON pre
CON post
CRA pre
CRA post
4-Hydroxy-3-methoxyphenylacetic acid
µg/mg creatinine
250
200
150
100
50
0
**
CON pre
CON post
CRA pre
CRA post

802

803 **Fig. 4**



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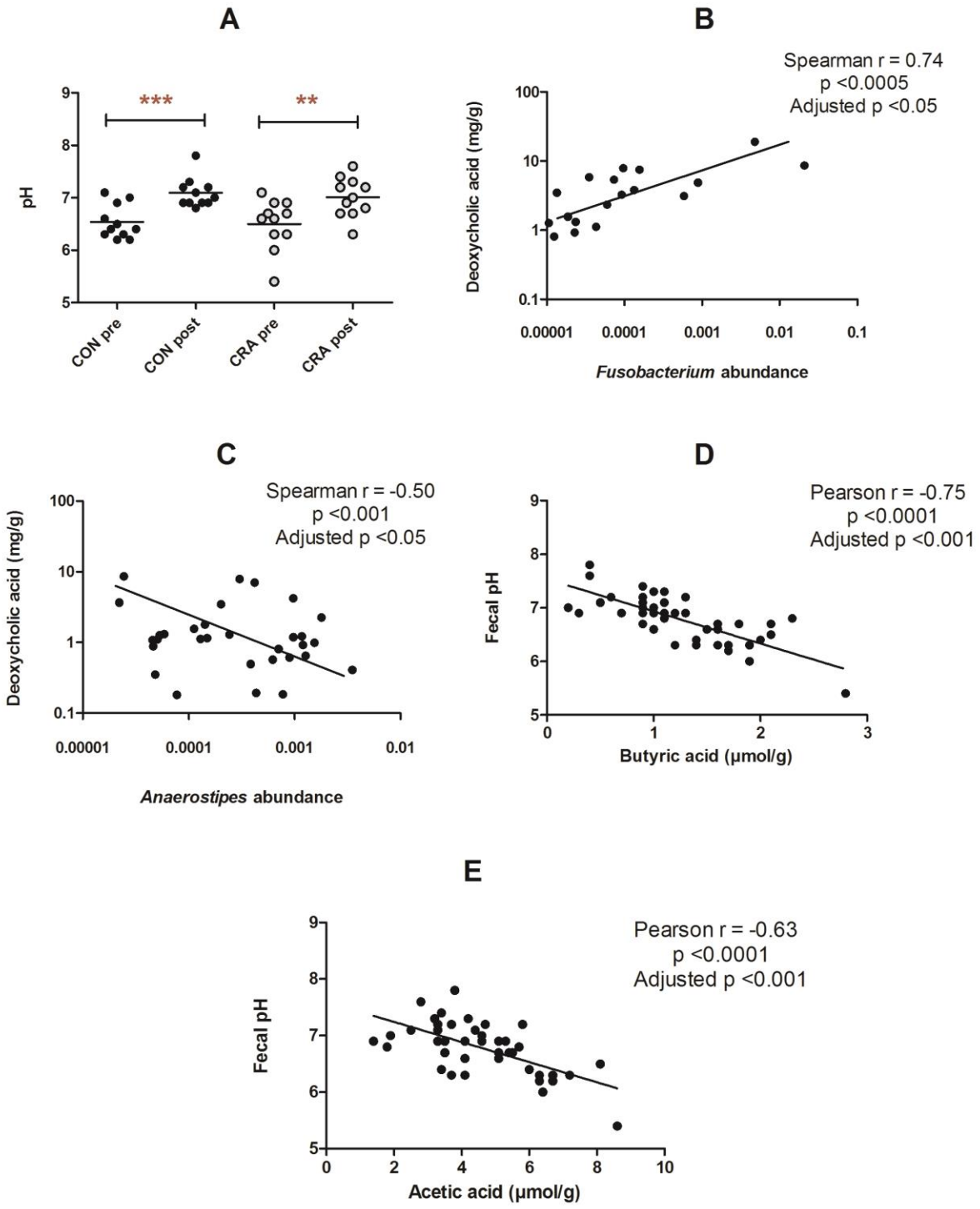
807

808 **Fig. 5**



809

810 **Fig. 6**

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Table 1. Baseline characteristics of participants

Variable	**Mean ± SD**		
	All (n=11)	Men (n=7)	Women (n=4)
Age (years)	39.2 ± 12.3	41.4 ± 12.4	35.3 ± 11.1
Height (cm)	171.6 ± 8.5	176.1 ± 4.9	163.8 ± 7.8
Weight (kg)	65.7 ± 8.9	70.7 ± 6.0	57.1 ± 5.8
BMI (kg/m^2)	22.2 ± 2.0	22.7 ± 1.9	21.3 ± 1.7
Waist circumference (cm)	81.5 ± 8.3	85.4 ± 8.1	74.6 ± 1.4
Hip circumference (cm)	93.7 ± 4.3	94.2 ± 4.8	92.7 ± 2.9
Diastolic blood pressure (mm Hg)	81.4 ± 7.9	83.6 ± 6.8	77.5 ± 8.0
Systolic blood pressure (mm Hg)	116.5 ± 12.6	122.4 ± 9.1	106.3 ± 11.0
Heart rate (beats per minute)	70 ± 13	70 ± 14	69 ± 11

Supplementary Material

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Text S1. Subject inclusion and exclusion criteria

Subject-inclusion criteria –

1. Age between 20-55 years
2. BMI between 18.5-29.9 kg/m^2
3. Bowel movement ≥3 times/wk
4. Consume ≥3 servings of fruits and vegetables daily on average
5. Willing to consume animal based diets for ≥10 days
6. Willing to consume freeze-dried whole cranberry powder
7. Willing to not take prebiotics and probiotics during the trial
8. Do not have allergic reaction to cranberries

Subject-exclusion criteria –

1. Colonoscopy 2 months prior to their enrollment or scheduled during the study
2. No antibiotic medications or drugs known to influence fecal microbiota were taken or used 3 mo before the study
3. History of a bilateral mastectomy
4. History of autoimmune disorders (rheumatoid arthritis, lupus, multiple sclerosis, vitiligo, psoriasis)
5. Consume <3 servings of fruits and vegetables daily one average
6. Gastrointestinal diseases, conditions, or medications influencing gastrointestinal absorption including active peptic ulcer disease or inflammatory bowel disease which will be found based on the self-report during the screening visit
7. Regular use of any acid-lowering medications (≥3 times/week)

8. Use of ≥14/wk serving of alcohol (168 oz beer, 56 oz wine, 14 oz hard liquor)
9. Intend to be pregnant, pregnancy, and breastfeeding
10. Infrequent (<3/wk) or excessive (>3/d) number of regular bowel movements
11. Active treatment for cancer (except non-melanoma skin cancer) and cardiovascular disease of any type ≤1 y
12. Having diabetes and/or receiving medications for diabetic condition, which will be found based on the self-report during the screening visit
13. Thyroid disease unstable or medication adjustments in past 6 months, which will be found based on the self-report during the screening visit
14. Values of standard blood biochemistries are unacceptable for the study based on study physician's discretion
15. Vegetarians and vegans, unwillingness or inability to consume animal-based foods including chicken, turkey, beef, eggs, cheese, other milk products, etc. Allergy to eggs or milk/dairy products.
16. Use anticoagulants, such as heparin, warfarin (Coumadin) in past 6 months
17. On or planning to be on a weight loss regimen
18. Regular use of any dietary supplements containing vitamins, minerals, herbal or other plant-based preparations, fish oil supplements (including cod liver oil) or homeopathic remedies; however, subjects who are willing to refrain from the use of these supplements at enrollment and throughout the entire study may be considered eligible
19. Use of monoamine oxidase inhibitors (MAOIs)
20. Glomerular filtration rate (GFR) <60 mL/min/1.73 m^2
21. Total cholesterol (TC) >250 mg/dL

22. Total triglycerides (TG) >300 mg/dL

Text S2. Waist and hip circumference measurements protocol

Abdominal (Waist) Circumference Measurement

- The volunteer is in a standing position.
- The volunteer is asked to hold up his gown.
- The examiner stands behind the volunteer and palpates the hip area for the right iliac crest.
- The examiner marks a horizontal line at the high point of the iliac crest and then crosses the line to indicate the mid axillary line of the body.
- The pants and underclothing of the volunteer must be lowered slightly for the examiner to palpate directly on the hip area for the iliac crest.
- The examiner then stands on the volunteer's right side and places the measuring tape around the trunk in a horizontal plane at this level marked on the right side of the trunk.
- The recorder walks around the volunteer to make sure that the tape is parallel to the floor and that the tape is snug, but does not compress the skin.
- The measurement is made at minimal respiration to the nearest 0.1 cm.

Buttocks (Hip) Circumference Measurement

- The volunteer stands erect with feet together and weight evenly distributed on both feet.
- The volunteer is holding up the examination gown.

- The recorder stands in back of the volunteer and gathers the side seams of the exam pants together above the hips and places the thumb in the fabric to make a fold.
- The recorder holds the folded sides of the pants snugly while the examiner squats on the right side of the volunteer and places the measuring tape around the buttocks.
- The tape is placed at the maximum extension of the buttocks.
- The recorder then adjusts the sides of the tape and checks the front and sides so that the plane of the tape is horizontal.
- The zero end of the tape is held under the measurement value.
- The tape is held snug but not tight.
- The examiner takes the measurement from the right side and calls it to the recorder

925 **Table S1.** Nutrition composition of freeze-dried whole cranberry powder

	1 g	30 g
Moisture (g)	0.2353	7.059
Dry matter (g)	0.7647	22.941
Protein (g)	0.032	0.96
Fiber (g)	0.4235	12.705
Calcium (g)	0.0006	0.018
Phosphorus (g)	0.0011	0.033
Magnesium (g)	0.0006	0.018
Potassium (g)	0.0076	0.228
Sulfur (g)	0.0004	0.012
Fat (g)	0.0243	0.729
Ash (g)	0.0168	0.504
Lignin (g)	0.0555	1.665
Starch (g)	0.2217	6.651
Proanthocyanidins (mg)	23.54	706.2
Hydroxycinnmate (mg)	1.52	45.6
Flavonols (mg)	4.63	138.9
Anthocyanins (mg)	2.79	83.7

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927

928 **Table S2.** An example of a one-day study menu including items to which either freeze-dried
929 cranberry or placebo powder were incorporated.
930

Meal	Food	FWCP or placebo powder (gram)
Breakfast	• Ham steak with cranberry syrup	4
Lunch	• Cheeseburger	6
	• Ginger ale spritzer	4
Dinner	• Cranberry Chicken Satay	4
	• Cranberry Lemonade	6
Snack	• Cranberry pudding	6
	• Ice cream	
Total		**30**

931 FWCP: Freeze-dried whole cranberry powder

932

Table S3. Nutrition composition of the example menu*

Nutrient	Values
Energy	2091 Kcal
Fats	94.5 g
Carbohydrates	217.1 g
Proteins	107.6 g
Saturated fats	50.2 g
Monounsaturated fats	28.5 g
Polyunsaturated fats	4.6 g
Cholesterol	495 mg
Fiber	9.8 g

*The actual amount of foods and nutrition compositions consumed by subjects were adjusted to meet their energy and nutrient needs.

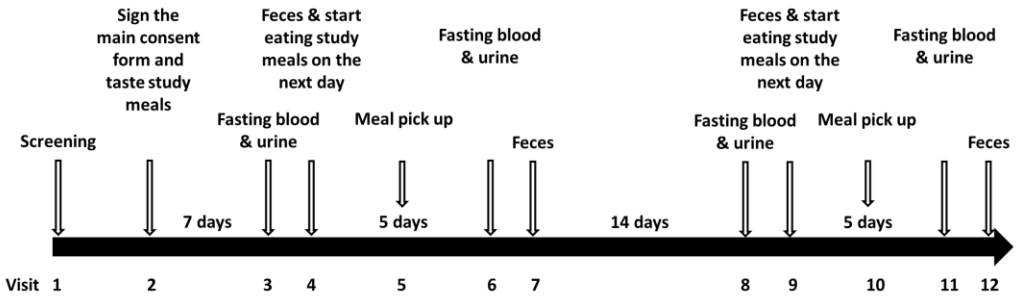

Fig. S1. Study timeline, including 12 study visits over 8 weeks.

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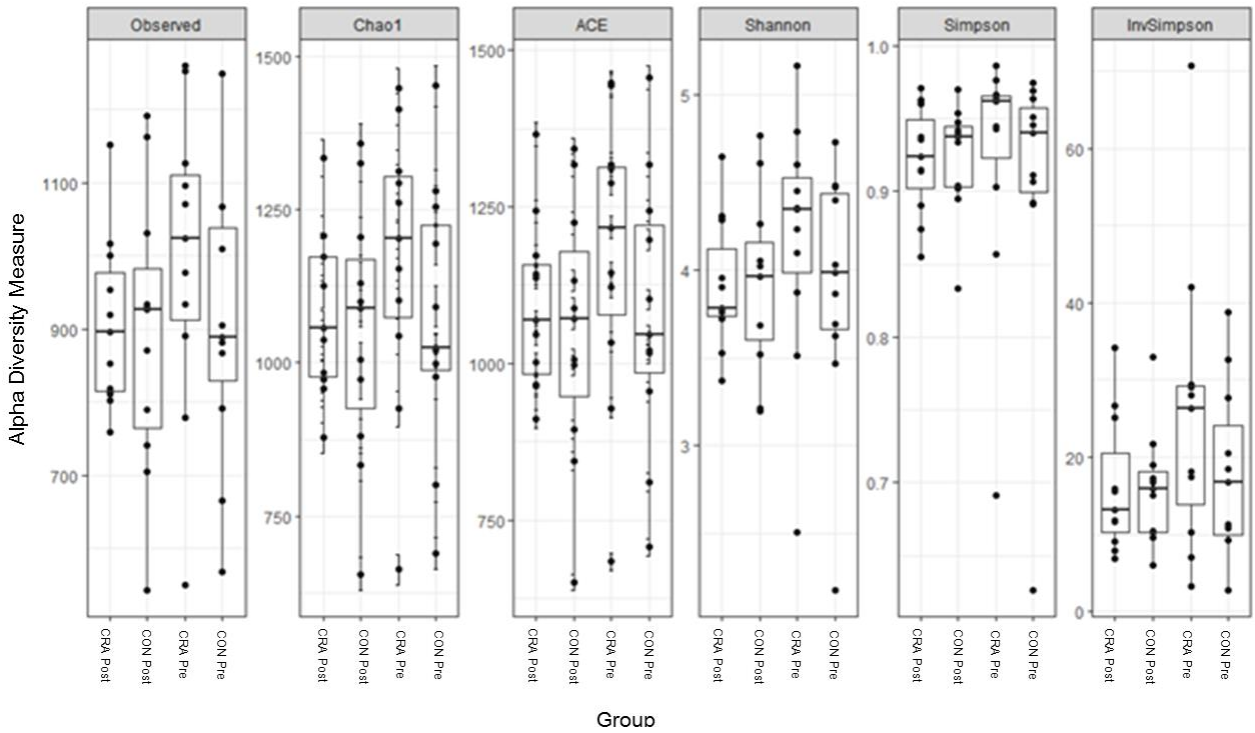

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943 **Fig S2.** Alpha-diversity indexes of stool collected from study participants before (pre) and after

944 (post) consuming control diet (CON) or cranberry diet (CRA) for 5 days.

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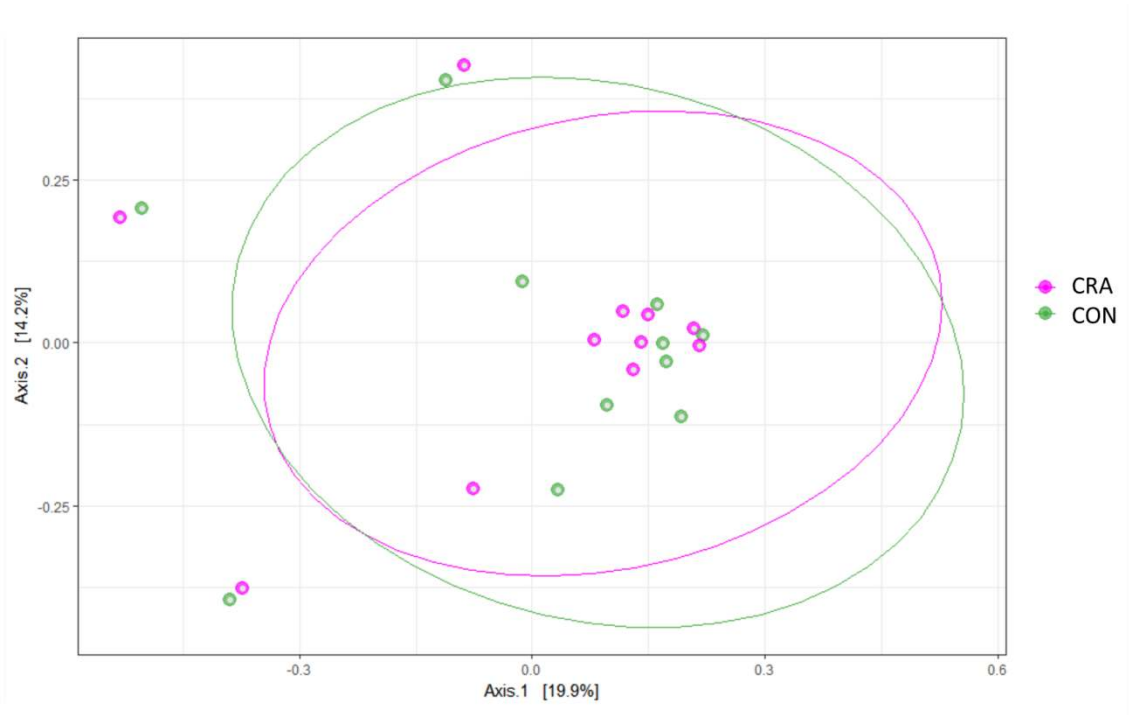

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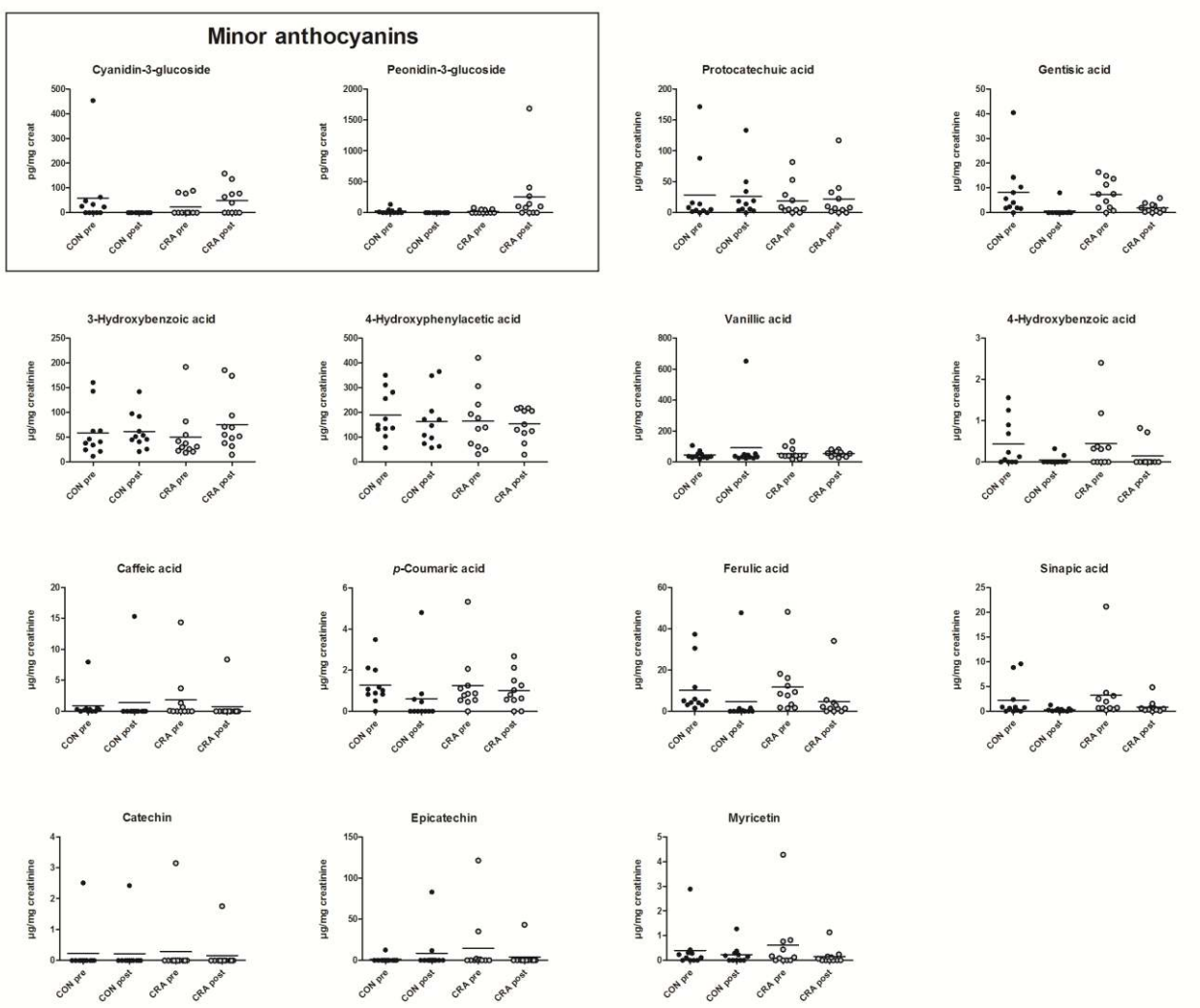
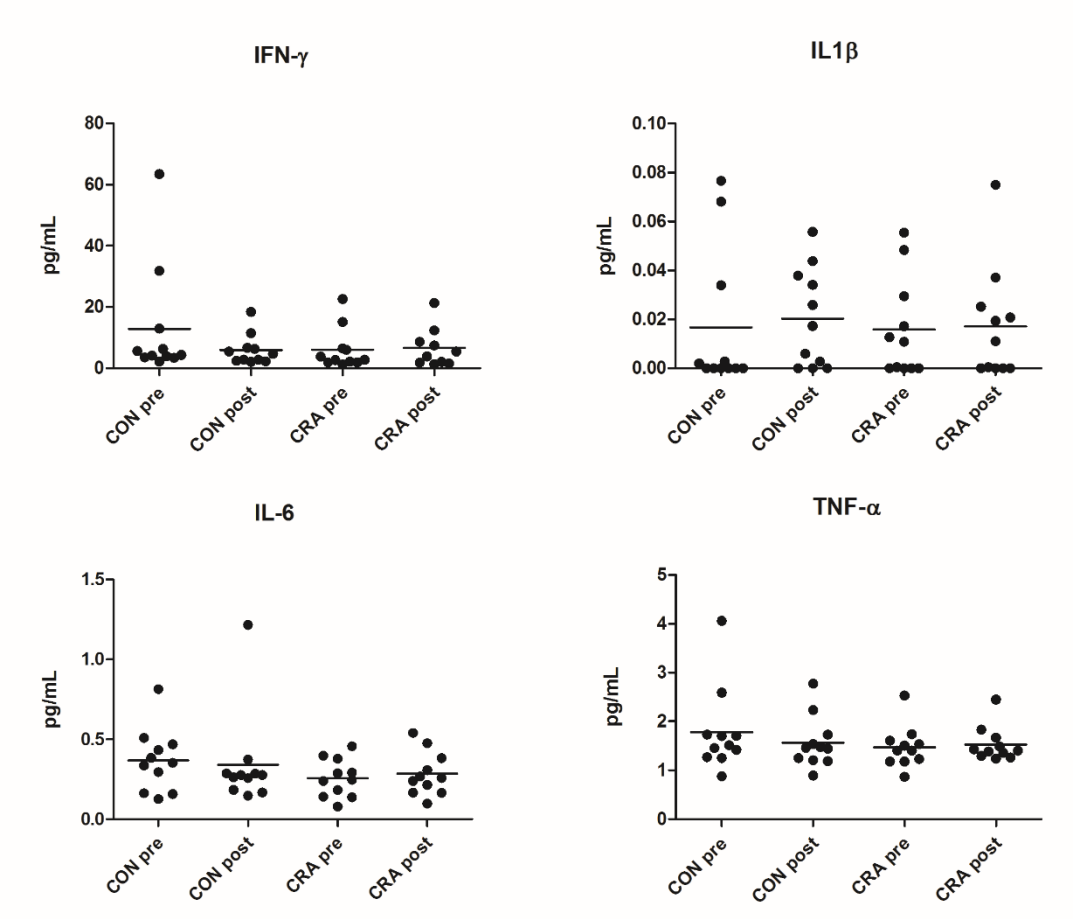


Fig. S4. Urinary levels of minor anthocyanins, phenolic acids, and flavonoids in study participants before (pre) and after (post) consuming control diet (CON) or cranberry diet (CRA) for 5 days.



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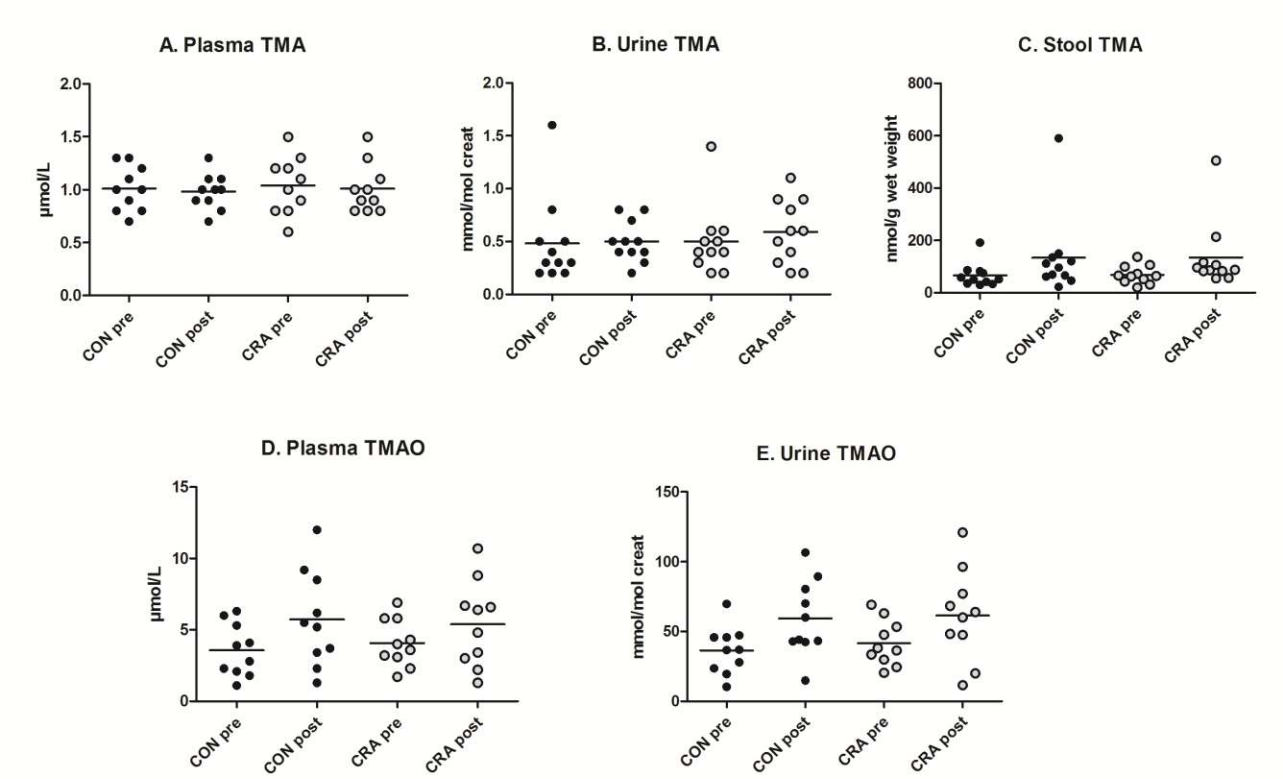
955 **Fig. S5.** Levels of plasma inflammatory biomarkers before (pre) and after (post) consumption of

956 the control diet (CON) and cranberry diet (CRA) for 5 days

957
958

959 **Fig. S6.** Trimethylamine (TMA) concentrations in plasma (**A**), urine (**B**) and stool (**C**);
960 trimethylamine-*N*-oxide (TMAO) concentrations in plasma (**D**) and urine (**E**).



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