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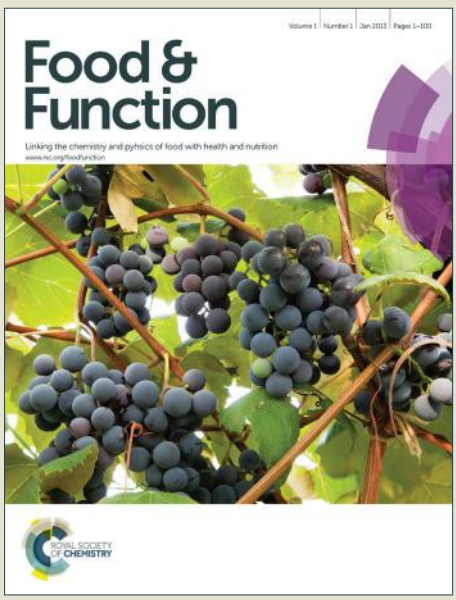

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Pomegranate extract induces ellagitannin metabolite formation and changes stool microbiota in healthy volunteers

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Abbreviations: EA ellagic acid, POM pomegranate, UA urolithin A

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Abstract

The health benefits of pomegranate (POM) consumption are attributed to ellagitannins and their metabolites, formed and absorbed in the intestine by the microbiota. In this study twenty healthy participants consumed 1000 mg of POM extract daily for four weeks. Based on urinary and fecal content of the POM metabolite urolithin A (UA), we observed three distinct groups: 1) individuals with no baseline UA presence but induction of UA formation by POM extract consumption (n=9); 2) baseline UA formation which was enhanced by POM extract consumption (N=5) and 3) no baseline UA production, which was not inducible (N=6). Compared to baseline the phylum *Actinobacteria* was increased and *Firmicutes* decreased significantly in individuals forming UA (producers). *Verrucomicrobia (Akkermansia muciniphila*) was 33 and 47-fold higher in stool samples of UA producers compared to non-producers at baseline and after 4 weeks, respectively. In UA producers, the genera *Butyrivibrio, Enterobacter, Escherichia, Lactobacillus, Prevotella, Serratia* and *Veillonella* were increased and *Collinsella* decreased significantly at week 4 compared to baseline. The consumption of pomegranate resulted in the formation of its metabolites in some but not all participants. POM extract consumption may induce health benefits secondary to changes in the microbiota.

Key Words: ellagitannins, human intervention study, microbiota, pomegranate, urolithin A

Introduction

In recent years, most health benefits associated with the consumption of pomegranate (POM) have been attributed to the presence of ellagitannins, mainly punicalagins and ellagic acid [1-4]. Ellagitannins are hydrolyzable tannins that contain galloyl and hexahydroxydiphenoyl groups, that produce ellagic acid upon hydrolysis [5]. Although POM ellagitannins are highly bioactive *in vitro*, they are not absorbed intact in the small intestine and undergo partial hydrolysis and spontaneous internal lactone formation to yield ellagic acid, which can be absorbed [6]. The remaining unabsorbed ellagitannins and ellagic acid are further metabolized to the dibenzopyranone-type urolithins A-D by the microbiota in the large intestine [7-10]. Our previous bioavailability studies have demonstrated that a small percentage of ingested phenolics circulate in plasma in the form of ellagic acid and urolithin A/B and their conjugated metabolites (dimethylellagic acid glucuronide; urolithin A/B glucuronide) and are excreted in urine [8].

The gut microbiota is an important contributor to human health [11] and has been implicated in the development of obesity and obesity-related diseases such as diabetes [12, 13] and cardiovascular disease [14]. The two most abundant bacterial phyla in humans and in mice are the *Firmicutes* (40–60%) and *Bacteroidetes* (20–40%) with lower abundance of *Actinobacteria*, *Fusobacteria*, *Proteobacteria* and *Verrucomicrobia* [15].

Previous investigations using batch culture fermentation of a fecal slurry has shown that the addition of POM by-product and punicalagins can alter the bacterial populations in mixed cultures [16]. POM addition significantly enhanced the growth of total bacteria, including *Bifidobacterium spp*. and the *Lactobacillus–Enterococcus* group, without affecting the growth of the *Clostridium coccoides–Eubacterium rectale* group and the *C. histolyticum* group while forming urolithins and short chain fatty acids [16]. Another publication compared the microbial

composition of stool samples cultured with the addition of EA. Stool samples with higher conversion of EA to UA compared to lower conversion of EA to UA contained higher abundance of *Clostridium coccoides* and no difference in *Bifidobacterium* [17]. In addition two species of *Gordonibacter* were identified to form urolithin A [18, 19].

In humans inter-individual differences in the production of urolithins from POM ellagitannins have been reported [20]. In about 5-25 percent of individuals urolithin metabolites are not detectable in urine after consuming pomegranate juice or extract [20]. It is our hypothesis that these differences are related to variation in the intestinal microflora.

We utilized a commercially available dietary supplement that is manufactured from the residual material after the first squeezing of the whole pomegranate fruit for juice production. Additional pressing and water extraction produces a liquid concentrate extract and further resin purification and drying produces a powder extract [21]. The pomegranate extract contains higher amounts of punicalagin A/B, punicalin and ellagic acid but a lower concentration of anthocyanins compared to juice [22]. The present study investigated the effect of this POM extract on the gut microbiota and formation of pomegranate metabolites after consumption of POM extract for 4 weeks. We also investigated the inter-individual differences in urolithin production by analysis of fecal and urine ellagic acid, urolithins, and their metabolites and whether differences in the microbiota prior to pomegranate ellagitannin consumption altered the formation of urolithins.

Methods

Study Participants

Twenty-seven subjects were recruited by advertisement in the local newspaper in the Los Angeles Area close to UCLA, based on inclusion and exclusion criteria. Six subjects withdrew

and one was lost to screening failure. Twenty healthy adults completed the study (9 healthy adult women and 11 healthy adult men). No adverse effects were reported. Subjects with a history of cigarette smoking in the past 5 years, history of bleeding disorders, inflammatory bowel diseases (Crohn's/ulcerative colitis), irritable bowel syndrome, gastrointestinal surgery within the past 2 years, diabetes, cardiovascular disease, hypertension, regular intake of NSAIDs, steroids, or other anti-inflammatory medications, use of antibiotics (other than topical) in the past 2 months and current use of dietary supplements, including probiotics and prebiotics, were excluded. Throughout the study participants were instructed not to consume pomegranate products, walnuts, or polyphenol-rich fruits (strawberry, raspberry, etc.) or juices drawn from a list used in prior studies. The study was carried out in accordance with the guidelines of the Human Subjects Protection Committee of the University of California, Los Angeles. All subjects gave written informed consent before the study began. The study was registered in ClinicalTrials.gov under the following identifier: NCT02370641.

Study Design

There were two study phases including a 2-week run-in period (low-flavonoid diet) and a 4-week intervention period. Subjects were instructed to take a daily dose of 1000 mg of the pomegranate extract (POMx®, POM Wonderful, Inc., Los Angeles), which delivers pomegranate polyphenols in an amount equivalent to about 8 oz of pomegranate juice. POM extract was developed to be used as a dietary supplement and has Generally Recognized as Safe status [23].

The POM extract contained 7% punicalagin A/B and 6.8% ellagic acid as determined by HPLC and LC-MS/MS [23] and a total phenolic content expressed as gallic acid equivalents of 680 µg/g as determined by the Folin-Ciolteu method [24]. According to the protocol, the study was closed after 20 participants completed the intervention.

Study Outcomes

The primary outcome of this intervention study was focused on the quantification of stool and urine pomegranate metabolites while for the secondary outcome changes in the stool microbiota were determined. Both outcomes were determined before (baseline) and after (4 week) the POM extract consumption.

Stool collection

Stools were collected before and after intervention. At each collection time, the entire fecal specimen was obtained. The specimen was placed in an approximately 3.8 liter plastic bag (Ziploc®, S.C. Johnson Co.) All air was pushed out of the bag as it was sealed, and the sample immediately stored at 4°C and delivered to the laboratory within 24 hours.

Fecal microbiological analyses

Stool specimens were placed into an anaerobic chamber and homogenized in a commercial blender and aliquots were provided for HPLC/LC-MS/MS analysis and DNA extraction. Fecal DNA was extracted using a commercial extraction system, (QIAamp® Stool DNA Extraction Kit, Qiagen, Valencia, CA). The quality of the DNA samples was confirmed using a Bio-Rad Experion system (Bio-Rad Laboratories, CA, USA).

MiSeq sequencing: Microbial sequencing was performed on the Illumina MiSeq next generation sequencing platform at the UCLA Sequencing and Genotyping Core Laboratory [25](Illumina, San Diego, CA). Briefly, the V3 and V4 region of 16S bacterial rDNA was amplified using the degenerate primer pair 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCW GCAG) and 805R (GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACH VGGGTATCTAATCC) with overhang adapters. The PCR conditions

consisted of 95°C 5min, 25 repetitions of 96°C 40 sec, 55°C 2 min, and 72°C 1 min, followed by final extension at 72°C for 7 min. PCR amplicons were purified using Qiagen PCR purification kit (Qiagen) and visualized on agarose gel, followed by EXOSAPIT (Affymetrix) to remove excess dNTPs and primers. The amplicons were normalized to 10 ng/µl concentration. KAPA HiFi Hot Start PCR Kit with dNTPs, 250 U (Illumina) was used to add the Illumina Nextera XT index on each end using a short PCR (95°C 5 min, [98°C 20 sec, 63°C 30 sec, 72°C 3 min] 5 cycles, 72°C 5 min). Agencourt AMPure XP Kit from (Beckman Coulter) was used to clean up unincorporated indexes and small fragments. An Agilent Bioanalyzer with a High Sensitivity chip was used to check for sizing and quality. Normalization across the samples was done using the SequalPrep Normalization plate kit (Invitrogen). The PCR products were pooled in equal volume and qPCR was performed to check quantitation using Illumina Genome Analyzer - KAPA SYBR FAST RocheLightCycler® 480 on Roche Lightcycler 480 instrument. The samples including indexed amplicons were loaded onto the MiSeq reagent cartridge and onto the instrument and analysis performed.

Data analysis

Automated cluster generation and paired-end sequencing with dual index reads was performed. Data was processed using the QIIME (Quantitative Insights Into Microbial Ecology) software package to identify the genus level of the metagenomic population showing the percentage of each genus present [26]. Briefly, the DNA sequence reads were demultiplexed according to nucleotide barcode and filtered for quality. Operational Taxonomic Units (OTUs) were picked based on sequence similarity within the reads and a representative sequence from each OTU was identified and assigned a taxonomic identity using the Greengenes sequence reference database [27]. The OTU sequences were aligned and a phylogenetic tree created. For each sample, diversity

metrics was calculated and the types of communities compared, using the taxonomic and phylogenetic assignments. Microbial diversity between different samples was assessed using beta diversity, and principal coordinate analysis (PCoA) plots generated to visually depict the differences between the samples [28].

Identification of pomegranate metabolites by high performance liquid chromatography and mass spectrometry

All solvents were HPLC grade from Fisher Scientific. Ellagic, formic and phosphoric acid and β-D- glucuronidase/ sulfatase (Helix pomatia H-5, G1512) were purchased from Sigma-Aldrich (St Louis, MO). Pure punicalagin A/B was isolated as previously reported [29] and urolithins A and B synthetized in our laboratory [8]. The composition of the pomegranate extract was analyzed by HPLC and LC-MS/MS. Method details are listed in electronic supplementary information. To determine fecal UA and EA blended stool material (50 mg) was mixed with 500 µL dimethylsulfoxide (DMSO), vortexed for 20 minutes and the mixture was centrifuged at 21,130xg. An aliquot (25 µL) of the supernatant was injected into the high performance liquid chromatograph (HPLC). Stool extracts were analyzed on a Surveyor HPLC system equipped with a diode array detector (DAD), scanning from 200 to 600 nm, and an autosampler held at 4°C (Thermo Finnegan, San Jose, USA). An Agilent Zorbax SB C-18 column, 250 x 4.6 mm, i.d. 5 µm was used and solvent elution consisted of a gradient system over 50 min of mobile phase A (0.1% phosphoric acid in H_2O) and mobile phase B (acetonitrile) at a flow rate of 0.75 ml/min. The following gradient was applied: 2-25% B (0-25 min), 25-40%B (25-32 min); 40-50%B (32-40 min) and 50-70%B (40-50 min). To determine the concentration of total urolithin in urine, samples were treated with glucuronidase/sulfatase as described previously for plasma[8]. Briefly, 200 µl urine was mixed with 500 U of glucuronidase/12.5 U of sulfatase in 100µL of

0.2M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid. The mixture was incubated at 37˚C for 45 min and centrifuged at 21130xg for 10min at 4°C. Supernatant was collected for HPLC analysis.

Statistical Analysis

This was an exploratory intervention study with 20 participants. Due to the lack of preliminary data we were not able to perform a power calculation to determine group size. Non parametric Wilcoxon rank sum test was used to compare continuous variables two groups, Chi-square or Fisher exact test was used to compare categorical variable and Spearman correlation coefficient was calculated among all variables by group and time. A $p \leq 0.05$ is considered statistically significant. Data management, variable transformations, and other statistical analyses were conducted using SAS 9.2 (Statistical Analysis System, Cary, NC, 2008).

Results

Demographics

Twenty participants completed the study. Participants with an UA content of urine and stool of <1 µg/g stool or <1 µg/mg creatinine in urine after 4 weeks of intervention were defined as non-producer and >1 µg/g as producer. 70% of participants were producers. The participants included nine healthy adult women and eleven healthy adult men with average age of 28.9 ± 8 years and body mass index (BMI) of 23.2 ± 3 kg/m^2 (Table 1). There was no difference in height, weight, BMI, gender, race, ethnicity or age between the producer and non-producer groups.

Urolithin A and ellagic acid metabolites in stool and urine after 4 weeks of administration of POM extract

Stool analyses revealed a large variability among participants in regards to the formation of POM metabolites (UA, EA, punicalin, punicalagin). At baseline, stool samples from 4 participants contained EA and 5 samples contained UA (Figure 1). Neither punicalagin A/B nor punicalin were present in baseline stools. Urinary UA at baseline ranged between 0 and 28 µg/mg creatinine. After 4 weeks of POM extract intake, fecal EA was found in 15 participants, punicalagin A/B in 15, punicalin in 13 and UA in 14 participants. The same 14 participants also had pomegranate metabolites detected in urine. Five participants were found to have UB in the stool sample after 4 weeks of POM extract intake while only one of five had UB in the stool at baseline (Table 2 supplementary online material). Stool samples from participants who were UA producers contained lower amounts of EA, while samples from non-producers had a higher EA content (Figure 1). The fecal EA and UA content ranged from 0-174 and 0-316.7 µg/g stool, respectively. The daily intervention of 1000 mg of POM extract provided 74 mg of punicalagin A/B, 66 mg of EA and 680 mg GAE. To compare the stool content of EA and UA with the consumed amount we estimated an average amount of stool per person per day of 1-2 kg stool volume and. the maximum fecal EA and UA was measured to be 174 mg/ kg stool and 316.7 mg/kg, respectively,

Effects of POM extract consumption on the gut microbiota

The abundance of bacterial phyla and genera were determined in stool samples collected at baseline and after 4 weeks of POM extract consumption. Data were analyzed to determine the change over time including all participants and separately for producers and non-producers. In

addition the difference between producers and non-producers at baseline and week 4 was analyzed. Including all participants, we observed a significant difference between baseline and week 4 in the phylum *Actinobacteria,* almost significant trend for *Proteobacteria* (p=0.053)(Figure 2) and a significant difference in abundance of *Verrucomicrobia* (*Akkermansia*) between producers and non-producers at baseline and week 4. In addition the phylum *Firmicutes* was significantly decreased and *Proteobacteria* significantly increased at week4 comparing producers and non-producers (Figure 2). The genera *Acetobacterium, Acidaminococus, Bifidobacterium, Butyrivibrio, Collinsella, Enterobacter, Erysipelothrix, Escherichia, Pseudobutyrivibrio, Serratia, Thermovenabulum and Veillonella* were significantly changed when including the data of all participants (Figure 3A). When analyzed separately by producers and non-producers we observed a significant increase in the genera *Butyrivibrio, Enterobacter, Escherichia, Lactobacillus, Prevotella, Serratia and Veillonella* and decrease in *Collinsella* in the producer group (Figure 3B). In non-producers the genus *Thermovenabulum*, a thermophilic environmental bacterium, was changed from baseline to week 4 significantly and *Actinobacillus* and *Bifidobacterium* tended to decrease with borderline significance (p=0.059 and 0.051, respectively) (Figure 3C). The comparison between producers and non-producers revealed that after 4 weeks the phyla *Verrucomicrobia, Proteobacteria* and *Firmicutes* were significantly different (Figure 2), and 11 genera (*Akkermansia, Anaerobranca, Anaerofilum, Butyricimonas, Candidatus Blochmannia, Desulfotomaculum, Desulfovibrio, Escherichia, Flavobacterium, Prosthecobacter, Rubritalea)* were changed significantly (Figure 4B). At baseline, the phylum *Verrucomicrobia* (Figure 2) and the genera *Akkermansia, Anaerobranca, Desulfonauticus, Desulfovibrio, Peptococcus, Polaribacter, Slackia and Tindallia* (Figure 4A) were significantly different between producers and non-producers. *Thermovenabulum*, *Prosthecobacter,*

Rubritalea, Desulfonauticus, Polaribacter, and *Tindallia* are known as environmental bacteria and of these, *Prosthecobacter* and *Rubritalea* are part of the *Verrucomicrobia* phylum. The significance of finding these genera in human stool samples is unclear.

In addition, we observed a non-significant trend of urinary UA being positively correlated with *Bacteroidetes* and negatively correlated with *Firmicutes* (Figure 5).

Discussion

The large molecular weight phenolic compounds in pomegranate are not absorbed but remain in the intestine, where they are metabolized by the gut microbiota and at the same time alter the composition of the microbiota. Ellagic acid and urolithins are the most commonly found metabolites of pomegranate ellagitannins resulting from microbial metabolism. This is the first investigation to evaluate global changes in the gut microbiota following the ingestion of a pomegranate extract. Data presented here demonstrate inter-individual differences in metabolizing pomegranate ellagitannins. We classified the observed responses into three distinct groups of individuals: 1) no baseline UA with induction of UA formation by POM extract; 2) baseline presence of UA, which was increased by POM extract consumption (producers) and 3) no baseline UA and UA not increased by POM extract consumption (non-producers). Among the 14 UA producers, 9 individuals did not show any UA in stool samples at baseline while 5 already contained low concentrations of UA in stool prior to POM extract consumption but all increased the production at the end of the period of POM extract consumption. Other dietary sources of ellagic acid such as walnuts, strawberries, raspberries and other nuts and berries [30-33] consumed prior to the study period may have contributed to the presence of urolithins at baseline but these were not consumed during the study period.

Six of the twenty participants (30%) did not have urolithin A in their stool or urine prior to POM extract intake and the UA formation was not stimulated by the POM extract consumption (non-producers). However, after 4 weeks of POM extract intake 50 % of the non-producers were found to have significant amounts of EA. It is possible that gut bacteria in those individuals with no detectable EA or UA were able to metabolize EA and UA to phenolic acids or to metabolize them further. When EA was present, it can be suggested that gut bacteria in those individuals were unable to convert EA to UA. The observed EA stool concentrations exceeded the estimated EA amount provided through the POM extract intervention suggesting that the POM extract ellagitannins most likely were broken down to EA and UA by gut bacteria. However, these hypotheses would need to be tested in future studies.

Our investigation focused on the formation of urolithin A. Studies by Tomas-Barberan et al [20, 34] demonstrated inter-individual differences in converting EA to UA, isourolithin A and/or urolithin B. Some individuals were unable to convert ellagic acid to UA in these studies. A higher level of urolithin B was found in individuals with chronic illness (metabolic syndrome or colorectal cancer) [20]. The same investigative group reported that individuals who produced urolithin had a much higher abundance of *Clostridium leptum* of the *Firmicutes* phylum than *Bacteroides/Prevotella*. Our data does not support those findings. The data in this study demonstrates that after four weeks of POM extract consumption there was a significant decrease of *Firmicutes* in UA producers compared to UA non-producers while *Prevotella* was increased significantly. Additional support for the importance of changes in the *Firmicutes* and *Bacteroidetes* ratio is corroborated by our observation that in UA producers there was a trend of negative correlation of *Firmicutes* to urine UA and positive correlation of *Bacteroidetes* to urine UA (Figure 5).

There is limited knowledge about bacteria that are able to break down pomegranate ellagitannins to ellagic acid and urolithins and further to smaller phenolic acids. Selma at al [19] identified two *Gordonibacter* species (*urolithinfaciens* and *pamelaeae*) that could produce urolithins from EA [19]. In addition, the same group found that *Clostridium coccoides* was increased while *Bifidobacterium* decreased in an individual producing UA and UA isomers compared to an individual with a lower capacity for urolithin production [17]. Our sequencing results did not identify any *Gordonibacter* species. However, differences in microbiota composition between the populations in Spain [19] compared to the U.S. based on a difference in dietary intake may be responsible.

Comparison of the composition of the stool microbiota demonstrated significant differences in the composition prior to the intervention as well as differences induced by the POM extract intake. The major difference between UA producers and non-producers was that the percent of *Verrucomicrobia (Akkermansia muciniphila*) was 33 and 47-fold higher in stool samples of UA producers compared to non-producers at baseline and after 4 weeks, respectively. Throughout the POM extract intervention the percent of *Akkermansia* did not change significantly. Another investigation that characterized changes in the microbiota after cranberry extract consumption in addition to a high fat/high sucrose diet in mice demonstrated a marked increase in proportion of the mucin-degrading bacterium *Akkermansia* in addition to a reduction in weight gain and increase in insulin resistance [35]. These data support the hypothesis that *Akkermansia* may play an important role in the breakdown of phenolic compounds in the intestine.

Comparing changes in the microbiota before and after the POM extract consumption separately in producers and non-producers demonstrated that the phylum *Proteobacteria* was significantly increased in producers and *Actinobacteria* significantly decreased in non-producers. On the

genus level we found that *Butyrivibrio, Collinsella, Escherichia, Enterobacter, Lactobacillus, Prevotella, Serratia, Veillonella* were increased significantly in producers at week 4 compared to baseline while *Bifidobacterium* was decreased (p=0.0508) in non-producers.

The phyla *Firmicutes, Bacteroidetes and Verrucomicrobia* have been demonstrated to be associated with obesity and chronic disease [36]. High concentrations of *Bacteroidetes* and low *Firmicutes* have been associated with lower body weight [37, 38]. The presence of the genus *Akkermansia* (*Verrucomicrobia*) has been demonstrated to inversely correlate with body weight and type-2 diabetes in rodents and humans [39, 40]. Several recent studies support the association of enterotypes *Bacteroides* and *Prevotella* with dietary habits [13, 41]. Animal protein and saturated fats were highly correlated with the *Bacteroides* enterotype and low meat intake and plant-based nutrition and high carbohydrates with *Prevotella* enterotype[41]. The enterotypes found in the subjects in this study were of the *Bacteroides* type with a small proportion of *Prevotella* enterotype. In the current study the abundance of *Prevotella* was significantly increased (2.5fold) in producers only. Chiu et al showed that the gut microbiota of people with lower body mass index also included a higher percentage of *Escherichia* (7.4 vs 12.%)[13]. In the present study, we observed an increase in *Escherichia* in producers after 4 weeks of POM extract intervention, which may contribute to the beneficial health effects of pomegranate.

Limitations to the study design included the low number of participants, relative short duration of intervention (4 weeks) and the use of one dose of POM extract. This study was of exploratory nature. Although the number of non-producers was low, we were able to determine significant changes. Future studies with a larger number of participants, longer intervention period and multiple doses of POM extract are recommended.

In summary, our data demonstrated that the composition of the microbiota determines whether individuals have the capability to produce phytochemical metabolites in their intestine. We also demonstrated that in 64% of the UA producers the ability to produce phytochemical metabolites was increased by 28 days of consumption of POM extract. POM extract may impact weight maintenance and insulin resistance by changing the ratio of *Firmicutes* to *Bacteroidetes* and increasing *Akkermansia* in the gut microflora. Further studies are needed to examine potential mechanisms by which POM extract may have beneficial effects on digestive health.

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ZL, DH, and SMF designed research and had primary responsibility for final content, SMH, RPL, PHS, GT and KC conducted research, SMH wrote paper, QYL reviewed data and manuscript, and CHT performed statistical analysis. None of the authors had a conflict of interest.

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Table 1: Demographics of study participants (N=20).

	Total (n=20)	Producers (n=14)	Non-Producers (n=6)	p-value
Age	28.9±8	29.1±7	28.5±11	0.91
Height	68±5	68±4	68±6	0.93
Weight	153±30	154±31	151±30	0.83
BMI	23±3	23±3	23±3	0.67
Female	9	6 (43)	3 (50)	0.99
Male	11	8 (57)	3 (50)	0.99
Race-White	14	10 (71)	4 (67)	0.99
Black	2	2 (14)	0 (0)	0.99
Asian	4	2 (14)	2 (33)	0.55
Ethnicity-Hispanic	5	3 (21)	2 (33)	0.61
Non-Hispanic	15	11 (79)	4 (67)	0.61

Data are mean±standard deviation. Numbers in parenthesis are percent.

Figure Legends

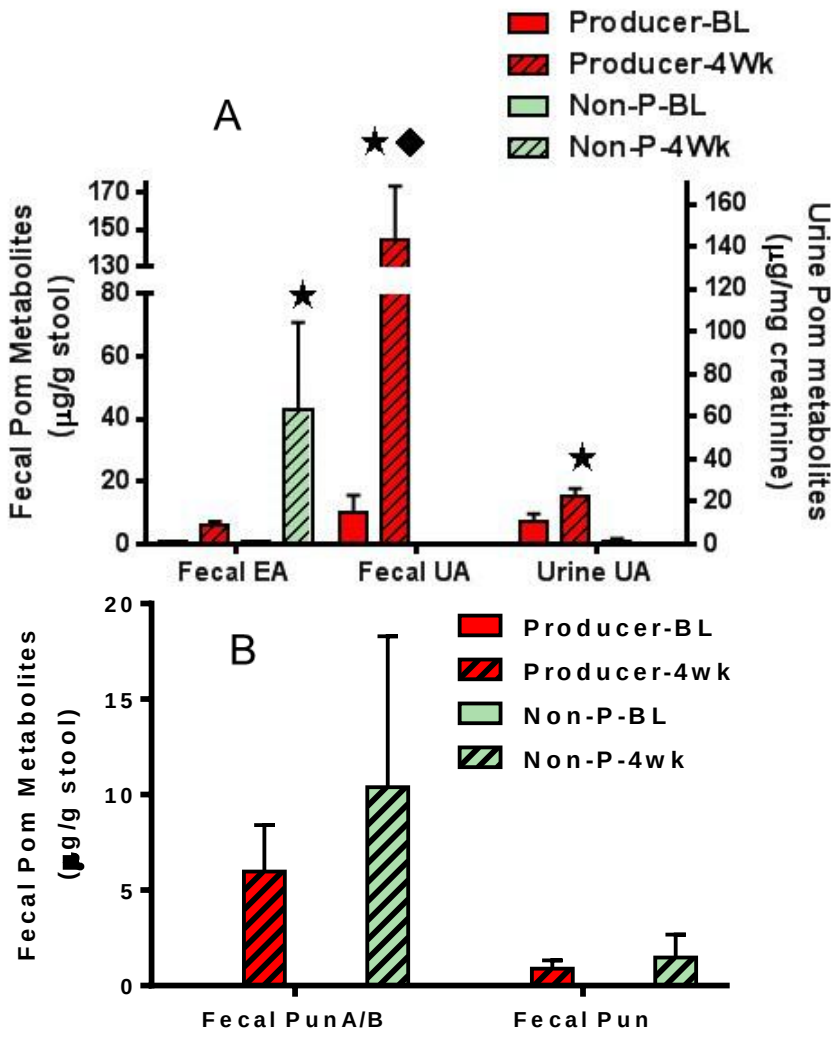
Figure 1. Urine (mg/g creatinine) and fecal (mg/kg wet weight) content of the pomegranate metabolites ellagic acid (EA) and urolithin A (UA) at baseline and after 4 weeks of POM extract consumption in producers (N=14) and non-producers (N=6) quantified by HPLC after enzyme treatment. A) Fecal ellagic acid, urolithin A and urine urolithin A; B) Fecal punicalagin A/B and punicalin. Data are mean±SEM. *significant difference between producers versus non-producers and ▪significant difference between baseline and week 4 ($p \leq 0.05$) as calculated by Student's t-test using SAS. Since baseline concentrations of punicalagin A/B and punicalin in Figure 1B were zero no statistical analysis was performed.

Figure 2. Abundance of phyla in all participants, producers and non-producers at baseline and week 4. Data is in percent of total phyla. *significant difference between baseline and week4 and ▪ between producers and non producers ($p \leq 0.05$) as calculated by non parametric Wilcoxon rank sum test using SAS.

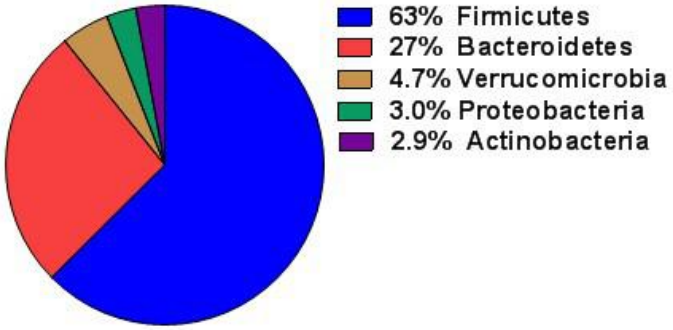
Figure 3. Statistical comparisons of gut bacterial profiles at the genus level. Plots showing differences in abundance of reads assigned to a given bacterial genus that were significantly different ($p \leq 0.05$) between baseline and week 4 for A) all participants (N=20), B) producers (N=14) and C) non-producers (N=6). The bar graph on the left side displays the mean proportion of sequences assigned to each genus. The dot plots on the right side display the differences in mean proportions between week 4 and baseline. Error bars on both sides of dots represent the 95% confidence intervals.

Figure 4 A. Statistical comparison of gut bacteria profiles at the genus level. Plots showing differences in abundance of reads assigned to a given bacterial genus that were significantly different ($p \leq 0.05$) between producers and non-producers at A) baseline and B) week 4. The bar graph on the left side display the mean proportion of sequences assigned to each genus. The dot plots on the right side display the difference in mean proportions between week 4 and baseline. Error bars on both sides of dots represent the 95% confidence intervals.

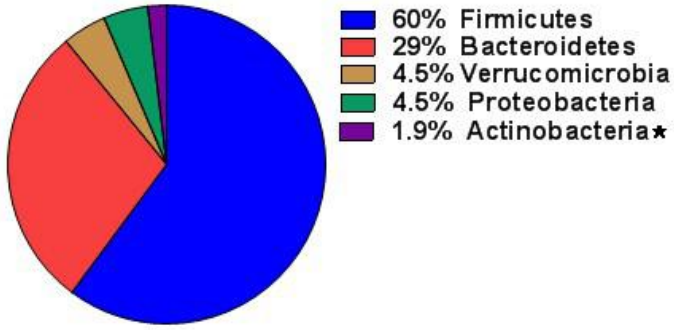
Figure 5. Correlation of urinary content of urolithin A to abundance of A) *Bacteroidetes* and B) *Firmicutes* in producers.



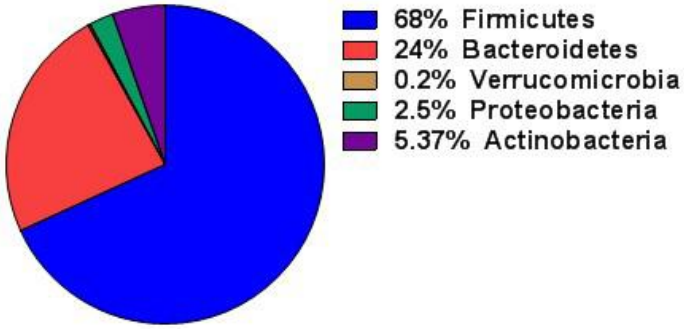
All Baseline



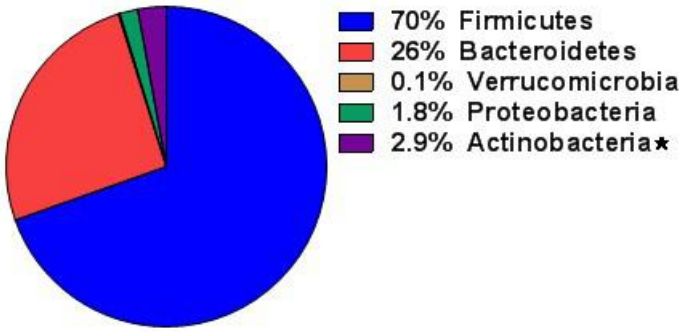
All Week4



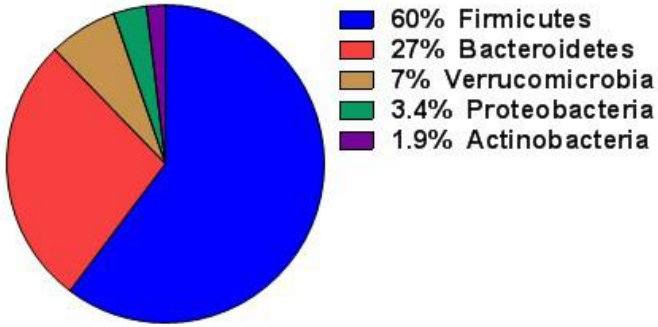
Non-Producer Baseline



Non-Producer Week4



Producer Baseline



Producer Week4